Zsolt Peter Nagy
Alex C. Varghese
Ashok Agarwal
Editors

In Vitro Fertilization

A Textbook of Current and Emerging Methods and Devices

Second Edition

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Editors
Zsolt Peter Nagy, MD, PhD
Reproductive Biology Associates – Prelude
Atlanta, GA, USA

Alex C. Varghese, PhD
Astra Fertility Group
Mississauga, ON, Canada

Ashok Agarwal, PhD
Cleveland Clinic
Cleveland, OH, USA

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Foreword

Exactly 40 years following the birth of the first IVF baby, Louise Brown, the extended and fully updated second edition of *In Vitro Fertilization* will see the light of day. This new edition, again edited by Drs. Nagy, Varghese, and Agarwal, convincingly captures the many advances in assisted reproduction technology, especially in the IVF laboratory. It is overwhelming to see the wealth of added knowledge since the publication of its first edition in 2012.

It is a privilege for me, as a clinician, to be asked to write the foreword for such an excellent and scholarly textbook, mainly focusing on the many developments in the ART laboratory. Reviewing the advances in ART since the late 1970s, it seems to me that the first few decades have been dominated by progress in the IVF clinic. Beyond any doubt, however, we all have wittnessed tremendous advances – away from the early days of "black box" fertilization and very early embryo development *in vitro* – in the laboratory during the most recent decade. I personally believe that a high-quality performance in the laboratory represents the key determinant for a successful IVF program. Quality management related to existing procedures, as well as the continued critical monitoring of possible benefits of novel technologies and the subsequent implementation of such new developments when proven beneficial, seem key determinants of a successful IVF laboratory.

The use of IVF continues to increase around the world, due to an ever-increasing global access to IVF care, the increasing use of ART in the treatment of different forms of infertility (like tubal disease, ovarian aging, or ovarian dysfunction), and – above all – the increasing central place of ART in conditions not directly related to infertility per se, such as the use of donor gametes in same-sex couples or singles; the cryopreservation of gametes, embryos, or gonadal tissue; genetic embryo testing in (often fertile) families suffering from genetic disease, and many others. Hence, the proportion of children born following ART will continue to increase in the years to come, and embryologists should be aware of their great responsibility toward the future health of ART offspring. Soon, at least one child in every primary school class will be generated by ART in the majority of countries worldwide.

Advances in the ART laboratory are everywhere, and they are all covered extensively in the 79 chapters of the current revised version of the book: the handling of sperm and oocytes, the many aspects of the process of fertilization, improved embryo culture media and culture conditions, and early embryo development. Moreover, many novel technologies have been developed for advanced embryo testing, and technologies are under way for automated and individualized embryo development assessment and handling (referred to by some as the "embryo hotel").

Novel information is being unraveled, stimulating embryologists to think outside the box, such as the potential influence of the patient and ovarian stimulation regimens on the quality of oocytes entering the laboratory. In addition, knowledge is accumulating regarding effects of the quality of embryos leaving the laboratory on implantation, miscarriage rates, and even pregnancy complications, perinatal morbidity, and possibly future health of IVF offspring.

Needless to say, I can highly recommend this authorative and comprehensive manual, primarily for everyone working in the IVF laboratory. Certainly, this book will also provide a wealth of useful information for everybody dealing with an IVF laboratory and may therefore fulfill the important role of bridging the gap between IVF clinicians and embryologists.

Bart C.J.M. Fauser MD, PhD, FRCOG
Emeritus Professor of Reproductive Medicine, University of Utrecht & University Medical Center Utrecht, The Netherlands

Preface

Ever since the first in vitro fertilization baby was born in Oldham, England, in 1978, the use of assisted reproductive technology (ART) to overcome infertility has increased exponentially with the simultaneous increase in the number of fertility centers in every part of the world. Since the first breakthrough, there have been several significant discoveries and improvements related to IVF technology, such as ICSI and vitrification (gametes, embryos, reproductive tissue), which has helped in increasing its efficiency severalfold.

The first edition of *Practical Manual of In Vitro Fertilization: Advanced Methods and Novel Devices* achieved an overwhelming success among clinical and laboratory professionals. It provided the most comprehensive update on all laboratory aspects of IVF, both theoretical and practical, in great detail and described several novel techniques that made the book also an outstanding reference for ART laboratory procedures.

The last six years has witnessed a steady stream of new discoveries and technological advancements in assisted reproduction laboratory methods and systems, such as automation, molecular testing, and gene editing, and with that growth, the necessity and demand for an updated new edition of this state-of-the-art textbook became readily apparent. For this second edition, the title shortened simply to *In Vitro Fertilization*, each section has been thoroughly revised and expanded to include the very latest evidence and practical guidelines, beginning with laboratory set-up, equipment, and management and moving on to detailed, thematic discussions of all relevant aspects of the IVF process: oocyte preparation and embryo culture methods, sperm processing and selection, insemination procedures, micromanipulation, embryo evaluation, grading and hatching, biopsy procedures, cryopreservation, and embryo transfer. The concluding sections present global perspectives on the regulation and licensing of ART laboratories; innovations, risks, and safety in ART; and molecular reproduction.

Every effort has been made to ensure that the information contained in this second edition is as up-to-date as possible, written by the most acclaimed and acknowledged professionals of our field – more than 160 experts in total, representing all continents of the world. Because of the wide range of topics and the comprehensive theoretical and detailed practical descriptions, this second edition is an ideal reference for all who are involved with assisted reproduction, including clinical embryologists, andrologists, reproductive endocrinologists, and scientists, regardless if one wishes to obtain a basic understanding or to digest an in-depth presentation. We ensure that this textbook will be a valuable resource for university students pursuing clinical embryology courses, too – chapters include clear learning objectives as well as review questions for self-guided study or group review.

We are thankful to Kristopher Spring, Senior Editor at Springer, for his ready support and advice, and Kevin Wright, Developmental Editor, for his continuous efforts in the day-to-day management of this large book project. We are grateful to Professor Bart Fauser for reviewing our book and authoring an outstanding foreword for the second edition. Furthermore, we are thankful to all of the exceptional contributors for sharing their knowledge and for being part of this great project.

Zsolt Peter Nagy, MD, PhD
Atlanta, GA, USA

Alex C. Varghese, PhD
Mississauga, ON, Canada

Ashok Agarwal, PhD
Cleveland, OH, USA

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Contributors

Ashok Agarwal, PhD
Department of Urology and American
Center for Reproductive Medicine
Cleveland Clinic
Cleveland, OH, USA
agarwaa@ccf.org

Martha Aguirre, PhD
Fertility Center of Las Vegas
Las Vegas, NV, USA
maguirre@ovationfertility.com

Robert John Aitken, PhD, ScD
Discipline of Biological Sciences and
Priority Research Centre in Reproductive Science,
School of Environmental and Life Sciences
The University of Newcastle
Callaghan, NSW, Australia
john.aitken@newcastle.edu.au

Carmela Albert Rodríguez, PhD
IVF Laboratory
IVI Valencia
Valencia, Spain

Lucía Alegre Ferri
IVF Laboratory
IVI Valencia
Valencia, Spain

Mina Alikani, PhD
Althea Science and IVFqc, ART Institute of Washington
New York, NY, USA

Akanksha Allahbadia, MD
A Rotunda – The Center for Human Reproduction
Mumbai, India

Gautam N. Allahbadia, MD
Indo Nippon IVF
Mumbai, India
drallah@gmail.com

Swati Allahbadia, MD
A Rotunda – The Center for Human Reproduction
Mumbai, India

Siti Nornadhirah Amdani
Nuffield Department of Obstetrics and Gynaecology
University of Oxford, Level 3, Women's Centre,
John Radcliffe Hospital
Oxford, UK

Matteo A. Avella, PhD
Shady Grove Fertility, Reproductive Science
Center of Pennsylvania, Department of Embryology
Wayne, PA, USA
matteo.avella@integramed.com

Doris J. Baker, PhD
Mercer University School of Medicine,
Basic Medical Sciences
Macon, GA, USA
dorisbkr@gmail.com

Basak Balaban, MSc
VKE American Hospital of Istanbul,
Assisted Reproduction Unit
Istanbul, Turkey
basakbalaban@superonline.com
basakb@amerikanhastanesi.org

Tom Beckitt, BS
Genea Biomedx
Sydney, NSW, Australia

Barry Behr, PhD, HCID
Stanford University, Reproductive
Endocrinology and Infertility
Sunnyvale, CA, USA
behr1@stanford.edu

Fabiola C. Bento, MBE
ANDROFERT, Andrology and Human Reproduction Clinic
Campinas, SP, Brazil
fabiola.bento@androfert.com.br

Diana P. Bernal, DMV
Biotech INC
Alpharetta, GA, USA
paticobernal@yahoo.com

Jutimala Bhattacharyya, MD
Dispur Poly Clinic IVF
Guwahati, India

Kia Borg, BA
Livio Fertilitetscentrum Göteborg
Göteborg, Sweden
kia.borg@fertilitetscentrum.se

Ching-Chien Chang, PhD
Reproductive Biology Associates – Prelude
Atlanta, GA, USA
changivf@yahoo.com

Alice A. Chen, PhD
GRAIL, Inc
Menlo Park, CA, USA

Hernest Institute for Reproductive Medicine
Sunnyvale, CA, USA
aliceannchen@gmail.com

Stephanie Cheung, BS
Weill Cornell Medicine, The Ronald O. Perelman and Claudia Cohen Center for Reproductive Medicine
New York, NY, USA
stc3001@med.cornell.edu

Ri-Cheng Chian, PhD
Center for Reproductive Medicine,
Shanghai 10th People's Hospital of Tongji University
Shanghai, China
rchian@126.com

Chak-Lam Cho
Department of Surgery, Union Hospital
Hong Kong, China
chochaklam@yahoo.com.hk

Stephen Chow, BA
Weill Cornell Medicine, The Ronald O. Perelman and Claudia Cohen Center for Reproductive Medicine
New York, NY, USA
stc3004@med.cornell.edu

Ana Cobo, PhD
IVI RMA
Valencia, Spain
Ana.Cobo@ivi.es

Jacques Cohen, PhD
Althea Science and IVFqc,
ART Institute of Washington
New York, NY, USA
jc@embryos.net

Giovanni Coticchio, BSc, MSc, MMedSci, PhD
9.Baby, Family and Fertility Center
Bologna, Italy
giovanni.coticchio@9puntobaby.it

Kevin Coward, BSC, PhD
Nuffield Department of Obstetrics and Gynaecology
University of Oxford, Level 3, Women's Centre,
John Radcliffe Hospital
Oxford, UK

Rachel Cutting, Bsc
Sheffield Teaching Hospitals NHS Foundation Trust,
Assisted Conception Department
Sheffield, South Yorkshire, UK
rachel.cutting@sth.nhs.uk

Shir Dar, MD
The IVF, Infertility and Reproductive Medicine Unit,
Department of Obstetrics and Gynecology,
Kaplan Medical Center
Rehovot, Israel

Madhab C. Das, PhD
Kolkata Global IVF
Kolkata, India
madhabcd@hotmail.com

Adam Dawood
Shady Grove Fertility, IVF
Rockville, MD, USA
adam.dawood@gmail.com

Shagufta Dawood, BS
Shady Grove Fertility, IVF
Rockville, MD, USA

Lucia De Santis, Bsc, MSc, PhD
IVF Unit, Department OB/GYN
San Raffaele Scientific Institute, Vita-Salute University
Milan, Italy
desantis.lucia@hsr.it

Tania R. Dias, MSc
American Center for Reproductive Medicine
Cleveland Clinic
Cleveland, OH, USA

Universidade da Beira Interior
Covilhã, Portugal

Department of Microscopy, Laboratory of Cell Biology
Institute of Biomedical Sciences Abel Salazar
and Unit for Multidisciplinary Research in Biomedicine,
University of Porto
Porto, Portugal

LAQV/REQUIMTE - Laboratory of Bromatology
and Hydrology, Faculty of Pharmacy
University of Porto
Porto, Portugal
taniairdias@gmail.com

Enver Kerem Dirican, PhD
Obstetrics and Gynecology, Center for Assisted Reproduction
Akdeniz University, Faculty of Medicine
Antalya, Turkey
keremdirican@gmail.com

Andrey V. Dolinko, MD
Women and Infants Hospital,
Obstetrics and Gynecology
Providence, RI, USA
andolinko@gmail.com

Marlena Duke, M.Sc., ELD
Reproductive Medicine Associates of New York
New York, NY, USA

Damayanthi Durairajanayagam, MD
Faculty of Medicine
Universiti Teknologi MARA, Sungai Buloh Campus
Sungai Buloh, Selangor, Malaysia

Paula Dwan, WHPN-BC. BS, MS
Third Party Reproduction
Boston IVF
Lexington, MA, USA
pdwan@bostonivf.com

Thomas Ebner, PhD
Kepler University, Department of Gynecology,
Obstetrics, and Gynecological Endocrinology
Linz, Austria
Thomas.Ebner@kepleruniklinikum.at

Thomas Elliott, BSc
Reproductive Biology Associates – Prelude
Atlanta, GA, USA
thomas@ivf.net

Marcos Meseguer Escrivá, PhD
IVF Laboratory, IVI Valencia
Valencia, Spain
Marcos.Meseguer@ivirma.com

Sandro C. Esteves, MD, PhD
ANDROFERT, Andrology and Human Reproduction Clinic
Campinas, SP, Brazil
s.esteves@androfert.com.br

Stephen Fiser
Althea Science and IVFqc, ART Institute of Washington
New York, NY, USA

Steven Fleming, PhD
Origio, Copenhagen, Denmark

University of Sydney, Sydney, Australia
sfleming@origio.com

Helena Fulka, PhD
Department of Biology of Reproduction
Institute of Animal Science
Prague, Czech Republic

Josef Fulka Jr, PhD
Department of Biology of Reproduction
Institute of Animal Science
Prague, Czech Republic
fulka.josef@vuzv.cz

Goral Gandhi, MA
Indo Nippon IVF
Mumbai, India

David K. Gardner, BSc (Hons), PhD
School of BioSciences
University of Melbourne
Parkville, VIC, Australia

Melbourne IVF
Melbourne, VIC, Australia
david.gardner@unimelb.edu.au

Forest C. Garner, MS
Fertility Center of Las Vegas
Las Vegas, NV, USA

Nicolás Garrido, PhD
IVI Foundation
Valencia, Spain
nicolas.garrido@ivi.es

Brant C. Gibson, PhD
ARC Centre of Excellence for Nanoscale
BioPhotonics (CNBP)
School of Science RMIT University
Melbourne, VIC, Australia
brant.gibson@rmit.edu.au

Antonia Gilligan, BA
Alpha Environmental
New York, NY, USA

Kathryn J. Go, PhD, HCLD
Embryology Laboratory, Boston IVF – The Maine Center
South Portland, ME, USA
KGo@bostonivf.com

Johan Guns, MSc
UZ Brussel
Brussels, Belgium
Johan.Guns@uzbrussel.be

Tatsuya Harada, MD, PhD
Kamaeda IVF Clinic Makuhari, Reproductive Medicine
Chiba, Japan
harada.tatsuya@kameda.jp

Thorir Hardarson, PhD
Livio AB
Stockholm, Sweden
thorir.hardarson@fertilitetscentrum.se

Ralf Henkel, B. ED, PhD, Habil
Department of Medical Bioscience
University of the Western Cape
Bellville, South Africa
rhenkel@uwc.ac.za

Christine Hennigan
Department of Molecular, Cellular
and Developmental Biology
University of Colorado
Boulder, CO, USA

Linda Hillis, BA
Donor Services, Boston IVF
Lexington, MA, USA
lhillis@bostonivf.com

Kenichiro Hiraoka, PhD
Kameda Medical Center,
Assisted Reproductive Technology Center
Chiba, Japan
hiraoka@oct.email.ne.jp

Julius Hreinsson, MSc, PhD
Mount Sinai Fertility
Toronto, Ontario, Canada
julius.hreinsson@gmail.com

Louise Hyslop, BSc, PhD
Newcastle Upon Tyne NHS Foundation Trust,
Newcastle Fertility Centre
Newcastle, UK
l.a.hyslop@newcastle.ac.uk

Romain Imbert
IVF Center CHIREC
Braine l'Alleud-Waterloo, Belgium

Evgenia Isachenko
University Maternal Hospital
Department of Obstetrics and Gynecology,
Cologne University
Cologne, Germany

Vladimir Isachenko, PhD
University Maternal Hospital
Department of Obstetrics and Gynecology,
Cologne University
Cologne, Germany
v.isachenko@yahoo.com

Tomonori Ishikawa, MD, PhD
Tokyo Medical and Dental University,
Comprehensive Reproductive Medicine
Tokyo, Japan
t.ishikawa.crm@tmd.ac.jp

Robert P. S. Jansen, MD CREI
Sydney IVF
Sydney, NSW, Australia

Ronny Janssens, BSc
Centre for Reproductive Medicine, UZ Brussel
Brussels, Belgium
Ronny.Janssens@uzbrussel.be

Kanna Jayaprakasan, PhD
Division of Obstetrics and Gynaecology,
Faculty of Medicine & Health Sciences
University of Nottingham
Nottingham, UK

Derby Fertility Unit
Royal Derby Hospital
Derby, UK
kanna.jayaprakasan@nhs.net
k.jayaprakasan@nottingham.ac.uk

Lars Johansson, Phd
NewLifeAid-Global
Torgny Segerstedts Alle21
Uppsala, Sweden
newlifeaidlj@gmail.com

Celine Jones
Nuffield Department of Obstetrics and Gynaecology
University of Oxford, Level 3, Women's Centre,
John Radcliffe Hospital
Oxford, UK

Hubert Joris
Vitrolife Sweden AB
Göteborg, Sweden
HJoris@vitrolife.com

Kiyotaka Kawai, MD
Kameda Medical Center, Assisted Reproductive
Technology Center
Chiba, Japan
kawai.kiyotaka@kameda.jp

Jonathan Kort, PhD, HCLD
Department of Reproductive
Endocrinology and Infertility
Stanford University
Stanford, CA, USA

Borut Kovačič, PhD
University Medical Centre Maribor,
Department of Reproductive Medicine
and Gynaecological Endocrinology
Maribor, Slovenia
borut.kov@ukc-mb.si
borut.kov63@gmail.com

Michelle Lane, PhD
Robinson Research Institute, School of Paediatrics and Reproductive Health, University of Adelaide
Adelaide, SA, Australia

Repromed, Dulwich, SA, Australia
michelle.lane@adelaide.edu.au

Christine Leary
Hull IVF Unit, East Riding Fertility Services
The Women and Children's Hospital, Hull Royal Infirmary
Hull, UK

Henry J. Leese
Hull York Medical School,
University of Hull, Hertford Building
Hull, UK

Don Leigh, PhD (UNSW)
Sydney IVF
Sydney, NSW, Australia

Juergen Liebermann, MS, PhD, HCLD
Fertility Centers of Illinois
Chicago, IL, USA
Juergen.Liebermann@integramed.com

Georgios Liperis, PhD, M Med Sci, MSc, BSc
Westmead Fertility Centre
Institute of Reproductive Medicine, University of Sydney
Westmead, NSW, Australia
gliperis@westmeadivf.com

Christa Mackenzie, BSc
Reproductive Science Center of the
San Francisco Bay Area
San Ramon, CA, USA

Roberta Maggiulli, PhD
Clinca Valle Giulia, Genera
Rome, Italy
maggiulli@generaroma.it

Peter Mallmann, MD
University Maternal Hospital
Department of Obstetrics and Gynecology,
Cologne University
Cologne, Germany

James Marshall, BAppSc (UTS)
Sydney IVF
Sydney, NSW, Australia

David Jareno Martinez
IVF Center CHIREC
Braine l'Alleud-Waterloo, Belgium

Ana D. Martins, MSc, PhD Candidate
Department of Urology and American Center
for Reproductive Medicine
Cleveland Clinic
Cleveland, OH, USA

Department of Microscopy,
Laboratory of Cell Biology and Unit
for Multidisciplinary Research in Biomedicine
Abel Salazar Institute of Biomedical Sciences (ICBAS),
University of Porto
Porto, Portugal

Steven J. McArthur, BSc
Sydney IVF
Sydney, NSW, Australia
stevenmcarthur@y7mail.com
Steve.mcarthur@sydneyivf.com

Marius Meintjes, PhD, HCLD
Frisco Institute for Reproductive Medicine
Frisco, TX, USA
mmeintjes@friscoinfertility.com

Rubina Merchant
A Rotunda – The Center for Human Reproduction
Mumbai, India

Kristine A. Milne, BS Biology
Shady Grove Fertility, IVF
Rockville, MD, USA
kristine.milne@integramed.com

Markus Montag, PhD
ilabcomm GmbH
Sankt Augustin, Germany
mmontag@ilabcomm.com

Dean E. Morbeck, PhD, HCLD, MBA
Fertility Associates
Auckland, New Zealand
dmorbeck@fertilityassociates.co.nz

Santiago Munné, PhD
CooperGenomics, a Cooper Surgical Company
School of Biosciences, University of Kent
Canterbury, UK
munne@reprogenetics.com

Maximillian Murtinger
NextClinic IVF Centers. Prof. Zech
Bregenz, Austria

Zsolt Peter Nagy, MD, PhD
Reproductive Biology Associates – Prelude
Atlanta, GA, USA
zsolt.peter.nagy@gmail.com

Laszlo Nanassy
Human Reproduction Institute
Budapest, Hungary
nanassyl@hotmail.com

Liesl Nel-Themaat, PhD
Department of Advanced Reproductive Medicine
University of Colorado Denver
Stapleton, CO, USA
liesl.nel-themaat@ucdenver.edu
lnthemaat@gmail.com

Queenie V. Neri, PhD
Weill Cornell Medicine, The Ronald O. Perelman
and Claudia Cohen Center for Reproductive Medicine
New York, NY, USA
qneri@med.cornell.edu

Willem Ombelet, MD, PhD
Genk Institute for Fertility Technology
Genk, Belgium

Akira Onishi, PhD
Transgenic Pig Research Unit
National Institute of Agrobiological Sciences
Tsukuba, Japan
onishi@affrc.go.jp

Yuval Or, MD
The IVF, Infertility and Reproductive Medicine Unit,
Department of Obstetrics and Gynecology,
Kaplan Medical Center
Rehovot, Israel

Gianpiero D. Palermo, MD PhD
Weill Cornell Medicine, The Ronald O. Perelman
and Claudia Cohen Center for Reproductive Medicine
New York, NY, USA
gdpalerm@med.cornell.edu

Pasquale Patrizio, MD, MBE, HCLD
Department of Obstetrics, Gynecology and
Reproductive Sciences
Yale University School of Medicine, Yale Fertility Center
New Haven, CT, USA
pasquale.patrizio@yale.edu

Nigel Pereira, MD
Weill Cornell Medicine, The Ronald O. Perelman
and Claudia Cohen Center for Reproductive Medicine
New York, NY, USA
nip9060@med.cornell.edu

Sonia Pérez Albalá
IVF Laboratory, IVI Valencia
Valencia, Spain

Anthony C. F. Perry, BSc, PhD
Laboratory of Mammalian Molecular Embryology,
Centre for Regenerative Medicine
University of Bath, Bath, UK

Department of Biology and Biochemistry
University of Bath, Bath, UK
perry135@aol.com

Adrianne K. Pope, PhD
Adrienne Pope Consulting
Brisbane, QLD, Australia
apopeconsulting@gmail.com

Catherine Pretty, BSc (bons), PhD
Nuffield Health, Woking Hospital,
Assisted Conception Unit
Woking, UK
catherine.pretty@gmail.com

Catherine Racowsky, PhD
Brigham and Women's Hospital, Obstetrics, Genecology,
and Reproductive Biology
Boston, MA, USA
cracowsky@partners.org

Gohar Rahimi, MD
University Maternal Hospital
Department of Obstetrics and Gynecology,
Cologne University
Cologne, Germany

Vanesa Y. Rawe
REPROTEC, Diagnóstico y Tratamiento Reproductivo
Buenos Aires, Argentina

Michael L. Reed, PhD
Fertility Center of New Mexico
Albuquerque, NM, USA
mleroyreed@gmail.com

Philipp Reineck, PhD
ARC Centre of Excellence for Nanoscale BioPhotonics
(CNBP), School of Science, RMIT University
Melbourne, VIC, Australia
philipp.reineck@rmit.edu.au

Don Rieger, BSc, PhD
LifeGlobal LLC, Research and Development
Guelph, ON, Canada
DonRieger@LifeGlobal.com

Laura Francesca Rienzi, BSc, MSc
G.E.N.E.R.A. Centre for Reproductive Medicine
of Valle Giulia Clinic
Rome, Italy
rienzi@generaroma.it

Rocio Rivera-Egea, MSc
Andrology Laboratory and Sperm Bank, IVIRMA Valencia
Valencia, Spain
Rocio.Rivera@ivi.es

Zev Rosenwaks, MD
Weill Cornell Medicine, The Ronald O. Perelman
and Claudia Cohen Center for Reproductive Medicine
New York, NY, USA
zrosenw@med.cornell.edu

Tammie Roy, PhD
Genea Biomedx
Sydney, NSW, Australia
tammie.roy@geneabiomedx.com

Amparo Ruiz, MD
IVI Valencia, Insitituto Valenciano De
Infertilidad (IVI Valencia)
Valencia, Spain
Amparo.Ruiz@ivi.es

Thamara Viloria Samochin, Phd MSc
IVF Laboratory, Instituto Universitario IVI Valencia
Valencia, Spain
thamara.viloria@ivi.es

Raul Sanchez, MD
Department of Preclinical Science
Faculty of Medicine, Universidad de La Frontera
Temuco, Chile

Sarthak Sawarkar
MedAnswers, University of Kent
Canterbury, UK
Sarthak.Sawarkar@coopergenomics.com

Heide Schatten, PhD
Department of Veterinary Pathobiology
University of Missouri-Columbia
Columbia, MO, USA
SchattenH@missouri.edu

Tim Schimmel, BA
Cooper Surgical
New York, NY, USA

Manesh Kumar Panner Selvam, PhD
American Center for Reproductive Medicine,
Cleveland Clinic
Cleveland, OH, USA

Bruce S. Shapiro, MD PhD
Fertility Center of Las Vegas
Las Vegas, NV, USA
bsshapiro@aol.com

Rakesh Sharma, PhD
Department of Urology and American Center
for Reproductive Medicine
Cleveland Clinic
Cleveland, OH, USA
sharmar@ccf.org

Shehua Shen, MD
Hernest Institute for Reproductive Medicine
Sunnyvale, CA, USA
shehuashen@gmail.com
shen.shehua@hernest.com

Zeev Shoham, MD
The IVF, Infertility and Reproductive Medicine Unit,
Department of Obstetrics and Gynecology,
Kaplan Medical Center
Rehovot, Israel
zeev1@mail.huji.ac.il

Sherman Silber, MD
Infertility Center of St. Louis
St. Louis, MO, USA

Sankalp Singh, MD
CRAFT Hospital and Research Centre
Kodungalloor, Kerala, India
sankalp489@gmail.com

Charalampos S. Siristatidis, MD
Assisted Reproduction Unit
"Attikon" Hospital, Medical School,
National and Kapodistrian University of Athens
Athens, Greece
harrysiri@yahoo.gr

Cecilia Sjoblom, MSc, PhD
Westmead Fertility Centre
Institute of Reproductive Medicine, University of Sydney
Westmead, NSW, Australia
cecilia.sjoblom@sydney.edu.au

Danielle G. Smith, PhD
Leeds Institute of Molecular Medicine,
University of Leeds, St James's University Hospital
Leeds, UK

Gary D. Smith, PhD
Departments of Obstetrics and Gynecology, Physiology,
and Urology, University of Michigan
Ann Arbor, MI, USA
smithgd@med.umich.edu

James J. Stachecki, PhD
Innovative Cryo Enterprises LLC
Linden, NJ, USA
jstachecki@gmail.com

James Stanger, PhD
FertAid Pty Ltd
Newcastle, NSW, Australia
office@fertaid.com

Astrid Stecher
NextClinic IVF Centers. Prof. Zech
Bregenz, Austria

Karl Sterzik, MD
Department of Reproductive Medicine
Christian-Lauritzen Institute, Ulm, Germany

Roger G. Sturmey, MD
Centre for Cardiovascular and Metabolic Research
Hull York Medical School, University of Hull,
Hertford Building
Hull, UK
rgs102@york.ac.uk

Qing-Yuan Sun, PhD
State Key Laboratory of Stem Cell
and Reproductive Biology
Institute of Zoology, Chinese Academy of Sciences
Beijing, China

Arne Sunde, PhD
Department of Clinical and Molecular Medicine
Norwegian University of Science and Technology
Trondheim, Norway
Trondheimivf@gmail.com

Jason E. Swain, PhD, HCLD
CCRM IVF Network
Lone Tree, CO, USA
jswain@ccrmivf.com

Lei Tan, PhD
Celmatix, Inc.,
New York, NY, USA

Hernest Institute for Reproductive Medicine
Sunnyvale, CA, USA
ltan0729@gmail.com

Jeremy G. Thompson, CNBP
ARC Centre of Excellence for Nanoscale BioPhotonics (CNBP)
The Robinson Research Institute, Adelaide Medical
School, The University of Adelaide
Adelaide, SA, Australia
jeremy.thompson@adelaide.edu.au

Alan R. Thornhill, Phd HCLD
Department of Gynaecology and Genetics Centre
The London Bridge Fertility
London, UK
arthornhill@aol.com

Akansha Tiwari, MD MSc
Yale University School of Medicine,
Obstetrics and Gynecology
New Haven, CT, USA
akansha.tiwari@yale.edu

Giles Tomkin, PhD
Althea Science and IVFqc
ART Institute of Washington
New York, NY, USA

Maria Traversa, BSc, Msc, (MED)
Sydney IVF
Sydney, NSW, Australia

Michael J. Tucker, PhD
Shady Grove Fertility, IVF
Rockville, MD, USA
mtucker@ivf.com

Filippo Maria Ubaldi, MD
G.E.N.E.R.A. Centre for Reproductive
Medicine of Valle Giulia Clinic
Rome, Italy
ubaldi@generaroma.it

Jonathan Van Blerkom, MD
Department of Molecular, Cellular and
Developmental Biology
University of Colorado
Boulder, CO, USA

Colorado Reproductive Endocrinology
Rose Medical Center
Denver, CO, USA
jonathan.vanblerkom@colorado.edu

Pierre Vanderzwalmen, Bio-Eng
IVF Center CHIREC
Braine l'Alleud-Waterloo, Belgium

NextClinic IVF Centers. Prof. Zech
Bregenz, Austria
pierrevdz@hotmail.com

Sabine Vanderzwalmen
IVF Center CHIREC
Braine l'Alleud-Waterloo, Belgium

Anne Vansteenbrugge
IVF Center CHIREC
Braine l'Alleud-Waterloo, Belgium

Alex C. Varghese, PhD
Astra Fertility Group
Mississauga, ON, Canada
alexcv2008@gmail.com

Sidney Verza Jr, MSc
ANDROFERT, Andrology & Human Reproduction Clinic,
Referral Center for Male Infertility
Campinas, SP, Brazil
labfiv@androfert.com.br

Ling Wang, BSc
Center for Reproductive Medicine
Shanghai 10th People's Hospital of Tongji University
Shanghai, China

Leslie Weikert, BSc
A.R.T. Institute of Washington, Inc.
Rockville, MD, USA

A.R.T. Institute of Washington, Inc.,
Rockville, MD, USA
leslieweikert@gmail.com

Klaus E. Wiemer, PhD
Poma Fertility
Kirkland, WA, USA
kewiemer@comcast.net
kwiemer@pomafertility.com

Matts Wikland, PhD
Department of Obstetrics and Gynecology
University of Gothenburg
Gothenburg, Sweden

Barbara Wirleitner, MSc, PhD
IVF Centers Prof. Zech, IVF Laboratory
Bregenz, Austria

Graham Wright, PhD
Reproductive Biology Associates
Atlanta, GA, USA

Wolfgang Würfel, MD
KCM (Kinderwunsch Centrum München) –
Fertility Center Munich
Munich, Germany
prof.w.wuerfel@ivf-muenchen.de

Liying Yan
Department of Obstetrics and Gynecology
Peking University Third Hospital
Beijing, China

Zhi-Yong Yang, MD
Center for Reproductive Medicine
Shanghai 10th People's Hospital of Tongji University
Shanghai, China

Marc Yeste, PhD
Department of Biology, Unit of Cell Biology,
Faculty of Sciences, University of Girona
Girona, Spain
marc.yeste@udg.edu

Peng Yuan, PhD
Department of Obstetrics and Gynecology
Peking University Third Hospital
Beijing, China

Abbreviations

ART	Assisted reproduction technology
DOHaD	Developmental Origins of Health and Disease
FET	Frozen embryo transfer
GM-CSF	Granulocyte-macrophage colony-stimulating factor
hsA	Human serum albumin
HTF	Human tubal fluid
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
IVC	In vitro culture
IVF	In vitro fertilization
LGA	Large for gestational age
TE	Trophectoderm
VOC	Volatile organic compounds

Journey of Human Gametes In Vitro: 1978 to 2018

Yuval Or, Shir Dar, and Zeev Shoham

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1.1 Introduction

The history of in vitro fertilization (IVF) and embryo transfer (ET) dates back as early as the 1890s when Walter Heape, a professor and physician at the University of Cambridge, England, who had been conducting research on reproduction in a number of animal species, reported the first known case of embryo transplantation in rabbits, long before the applications to human fertility were even suggested.

In 1932, *Brave New World* was published by Aldous Huxley. In this science fiction novel, Huxley realistically described the technique of IVF as we know it. Two years later Pincus and Enzmann, from the Laboratory of General Physiology at Harvard University, published a paper in the *Proceedings of the National Academy of Sciences of the United States of America*, raising the possibility that mammalian eggs can undergo normal development in vitro. However, it was not until 1959 that indisputable evidence of IVF was obtained by Chang [1], who was the first to achieve births in a mammal (a rabbit) by IVF. The newly ovulated eggs were fertilized, in vitro by incubation with capacitated sperm in a small Carrel flask for 4 h, thus opening the way to assisted procreation.

Professionals in the fields of microscopy, embryology, and anatomy laid the foundations for future achievements. The recent rapid growth of IVF-ET and related techniques worldwide is further supported by the social and scientific climate, which favors their continuation.

Through the years, numerous modifications have been made in the development of IVF-ET in humans: refinement of fertilization and embryo culture media, earlier transfer of the embryo, improvements in equipment, use of a reduced number of spermatozoa in the fertilization dish, and embryo biopsy, among others.

The purpose of this chapter is to acknowledge those who initiated new steps in the development of the treatment protocols and techniques that we now use facilitating such simple and promising IVF-ET procedures.

1.2 Development of IVF

In 1965, Robert Edwards together with Georgeanna and Howard Jones at The Johns Hopkins Hospital in the USA attempted to fertilize human oocytes in vitro [2]. It took another 8 years until the first IVF pregnancy was reported by the Monash research team of Professors Carl Wood and John Leeton in Melbourne, Australia. Unfortunately, this resulted in early miscarriage [3]. Another step toward the goal of IVF was noted by Steptoe and Edwards who published a report on an ectopic pregnancy following transfer of a human embryo at the late morulae/early blastocyst stage [4]. However, a breakthrough and a huge achievement were noted in 1978 when the first ever IVF birth occurred in Oldham, England, on July 25, 1978. This birth was the result of the collaborative work of Patrick Steptoe and Robert Edwards [5]. A year later, the first IVF birth was noted in Australia by the joint Victorian Monash-Melbourne team that occurred at The Royal Women's Hospital [6]. This was followed by the announcement of Howard and Georgeanna Seegar Jones about the delivery of the first IVF baby in the USA, in 1981. This first IVF birth in the USA was achieved with the use of hMG for ovarian stimulation. The improvement of technology leads to the report of Jacqueline Mandelbaum with Dan Szollosi describing the microstructures of the human oocyte, which became known as "oocyte dysmorphia" [7].

To improve the results of the procedure, efforts were made in optimizing the stimulation protocols, improving the culture media, improving aspiration technique and holding of the oocytes, and improving fertilization techniques. The second part of this chapter describes the six fields that were developed in parallel, enabling to improve the pregnancy rate. These fields are stimulation protocols; culture media; aspiration technique, handling of the oocyte, and embryo replacement; improved fertilization; cryopreservation and storage of embryos and oocytes; and future manipulation of the oocytes and embryos.

1.3 Stimulation Protocols

Earlier IVF pregnancies were achieved from unstimulated ovulatory cycles, and the success rate remained low. It was later on suggested that in order to improve the fertilization and pregnancy rate, it might be possible to aspirate more than one egg, and this can be achieved during a cycle in which the ovary is stimulated by endogenous or exogenous gonadotropins. During the years, several protocols were developed, and each of it was tested in relation to the drug influence on the oocytes. Alan Trounson and his colleagues introduced the use of clomiphene citrate and hMG in the treatment protocols [8]. It was later on demonstrated that GnRH agonists can be used to eliminate premature luteinization and control ovarian stimulation [9].

It was Porter and his colleagues who were the first to use GnRH agonists in IVF treatment [10]. Three major protocols using GnRH agonist were developed: the long, short, and ultrashort protocols. Several years later following numerous studies, it was found that the long protocol gave the best results in terms of pregnancy rate.

The administration of GnRH agonists resulted in "flare-up" of gonadotropins from the hypophysis. In some women, this resulted in the development of follicular cyst. In order to reduce the scale of gonadotropin section following the administration of GnRH agonist, it was suggested to give GnRH agonist during the luteal phase, when serum progesterone is high, or under progesterone administration or during the oral contraception pill. Gonen, Jacobson, and Casper pioneered the use of combined oral contraceptives for follicle synchronization and cycle scheduling in IVF [11].

Following the development of the various stimulation protocols, it was noted that there is a possibility that the

steroid production during the luteal phase was not optimal, and it was Casper and his colleagues who were the first to describe the use of low-dose hCG for support of the luteal phase in ART cycles [12].

In order to simplify the stimulation protocol and to shorten it, efforts were invested in developing the GnRH antagonist. Rene Frydman was the first to report the use of the GnRH antagonist, Nal-Glue, to prevent premature LH rise and progesterone in controlled ovarian hyperstimulation treatment [13]. This specific drug was not developed further due to the side effects. Following further efforts, the third generation of the GnRH antagonist was developed, and the first established pregnancy using recombinant FSH and GnRH antagonist was reported in 1998 by Itskovitz-Eldor and his colleagues [14].

The increasing needs in infertility treatment created the needs to develop new sources for gonadotropins. Recombinant FSH became available in 1992, and very soon after, pregnancy was established using recombinant drug for ovarian stimulation [15, 16].

Following the development of the technology to produce recombinant FSH, it became only a matter of time until the recombinant LH and hCG became available. Pregnancy after treatment with three recombinant gonadotropins was reported by Agrawal and colleagues in 1997 [17].

Final oocyte maturation is usually achieved by hCG administration 30–36 h prior to the ovum pickup. Several additional developments were noted when Emperaire et al. published their observation that the final stage of ovulation induction can be induced by endogenous LH released by the administration of an LHRH agonist after follicular stimulation for in vitro fertilization [18]. Gonen et al. in their publication have shown that GnRH agonist can be utilized for final oocyte maturation in GnRH antagonist protocols and reducing the risk of ovarian hyperstimulation syndrome [A].

Double stimulation during the follicular and luteal phases has been introduced by Kuang et al. In this protocol, ovarian stimulation and egg retrieval are followed by a second stimulation and retrieval utilizing the antral follicles left after the first stimulation cycle in order to increase oocyte yield. For the first stage, clomiphene citrate and letrozole were used for ovarian stimulation in conjunction with human menopausal gonadotropin (hMG). GnRH agonist was utilized for final oocyte maturation before oocyte retrieval. For the second-stage ovarian stimulation, hMG and letrozole were administered daily from the day of, or the day after, oocyte retrieval. Letrozole administration was stopped when the dominant follicles reached diameters of 12 mm, and medroxyprogesterone acetate 10 mg was added beginning on stimulation day 12 to avoid oocyte retrieval during menstruation. GnRH agonist was utilized again for final oocyte maturation. All the embryos were vitrified and transferred later during a thawed cycle [D].

The abovementioned developments summarize 40 years of gradual development in production of drugs and methods of administration.

In addition, a new technology was developed, which involved in vitro maturation of the oocyte (IVM). Chian et al. demonstrated that hCG priming prior to immature oocyte retrieval in women with PCO increases the maturation rate and produces high pregnancy rates of 40% per IVM started cycle [19].

It seems today that the various protocols and the various drugs available for ovarian stimulation enable the treating physicians to individualize treatment to the specific patient.

1.4 Culture Media

Optimal culture medium, which reflects the changing needs of the developing embryo, is of importance for the success of IVF treatment. The environment of the oviduct/uterus changes dramatically as the zygote/embryo travels. The culture media need to meet the metabolic needs of preimplantation embryos by addressing energetic and amino acid requirements in a stage-specific manner.

The first advanced culture medium was introduced in 1980 [20]. In 1985, Quinn and Warnes published a formula entitled human tubal fluid (HTF) that mimics the in vivo environment to which the embryo is exposed [21]. Gardner introduced sequential media and blastocyst transfer, which now greatly assists in the move to single embryo transfer [22, 23].

The development of a completely chemically defined protein-free embryo culture medium and the births of the first batch of babies generated from the fertilization of eggs collected and inseminated in the medium using spermatozoa also prepared in the same protein-free medium in both conventional IVF and ICSI were introduced in 2000 [24], and since then the medium remained almost the same.

1.5 Aspiration Technique and Handling of the Oocyte and Embryo Replacement

Several techniques for oocyte aspiration have been developed to obtain the largest number of mature oocytes with the least risk to the patients causing minimal damage to the ovary. This was stated with laparoscopic retrieval introduced by Steptoe and Edwards in 1978 and laparotomy for infertility-related conditions [25].

Susan Lenz and Jurgen G Lauritsen examined and proposed the transabdominal transvesical oocyte aspiration using an ultrasound-guided needle [26].

A year later, Gleicher and his group reported the first vaginal egg retrieval using an abdominal ultrasound [27].

Matts Wikland together with Lars Hamberger and Lars Nilsson in Gothenburg, Sweden, described the possibility of using a vaginal sector scanner (transvesical or transvaginal technique) for oocyte aspiration [28]. At the same time, Strickler et al. [29] described embryo transfer using abdominal ultrasound guidance. This technique gained additional support from the work done by Feichtinger et al. [30], and it remained the main technique for egg collection until today.

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1.6 Improved Fertilization

In the process of fertilization, it was first reported by Trounson and his group that there is a need for a delay between oocyte collection and insemination to allow oocytes collected to complete maturation [31]. In parallel, the possibility of using the natural environment of the human tube for the process of fertilization and development of the embryo leads to the development of the gamete intrafallopian tube (GIFT) procedure, and it was Asch and his colleagues, in 1984, who were the first to report a pregnancy following translaparoscopic GIFT procedure [32].

Much effort was invested trying to improve fertilization when this could not occur naturally when the egg and the sperm were introduced at the Petri dish in the laboratory. These efforts were started when researchers try to fertilize human oocytes by microinjection of a single sperm under the zona pellucida [33], which was followed by the report of a healthy delivery using the same technique in 1988 by Ng and his colleagues [34]. Further achievement was noted when pregnancy was obtained from micromanipulation using zona drilling or mechanical partial zona dissection [35]. However, a breakthrough was achieved when Palermo and his colleagues from the group in Brussels report of the first pregnancy after intracytoplasmic sperm injection (ICSI) [36].

In order to improve fertilization results after repeated IVF failure or at advanced maternal age, it was suggested to use the cytoplasmic transfer technique. This is based on an assumption that a vital molecule, such as ATP or cell cycle-related kinase, or an organelle, such as mitochondria, is deficient at a critical stage in early development.

The technique aims to improve egg and embryo quality by the donation of ooplasm from supposedly fertile oocytes to patients whose cells are of poorer quality. Transfer of enucleate cytoplasm has been used, and Cohen et al., in 1997, were the first to report on a birth after such a procedure [37].

1.6.1 Embryo Selection

Embryo selection is a key point in IVF success. Since our ability to select genetically normal embryo was limited, invasive and noninvasive techniques have been developed.

1.6.2 Noninvasive Selection

In their pioneering work, Wong et al. have shown a correlation between time-lapse image analysis and gene expression profiling. The timing of events during embryo formation is positively correlated to blastocyst formation [E].

The utilization of proteomics to analyze embryo's euploidy was suggested by McReynolds et al. (2011). They revealed that the presence of lipocalin-1 is increased in the secretome of aneuploid human blastocysts. The data were obtained through mass spectrometry (MS) and subsequently confirmed through enzyme-linked immunosorbent assay (McReynolds 2011) [F].

1.6.3 Invasive Selection

A new era in the field of IVF, i.e., preimplantation genetic diagnosis (PGD), was open with the announcement of Handyside and his colleagues who first reported on the biopsy of human preimplantation embryos and sexing by DNA amplification [49]. This was followed by the report of pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification [50]. Still in the same field of PGD, Verlinsky was the first to report on the possibility to do a polar body biopsy, transfer the embryo, and achieve pregnancy [51].

Embryo biopsy at different stages (polar body biopsy, cleavage stage (Day 3), or blastocyst) has been used for single gene mutation (preimplantation genetic diagnosis (PGD)) or for comprehensive chromosome screening (preimplantation genetic screening (PGS)). The clinical pregnancy rate for single screened blastocyst was comparable to two unscreened blastocysts dropping the twins rate to 0 (Forman et al. 2013) [G]. Although this technique is promising, it is not the routine practice till date, and further studies are needed.

1.7 Development of Cryopreservation and Storage of Embryos and Oocytes

In 1983, the group of investigators at the Monash IVF Centre reported on the first human pregnancy and birth following cryopreservation, thawing, and transfer of an eight-cell embryo. This embryo freezing technique was developed in Cambridge, England, on cattle, and with minor adaptations, it was adjusted to humans [38]. Few years later, in 1990, Gordts and his colleagues reported on the first successful human cleavage-stage embryo vitrification followed by a successful delivery [39]. However, until this period of time, it was difficult to cryopreserve oocytes, and only in 1999, the birth following vitrification of human oocyte was reported by Kuleshova et al. [40]. During the same year, Porcu and her colleagues reported on the first birth from cryopreserved oocytes and testicular sperm [41]. A major development was noted when Bedaiwy et al. reported in 2006 on the successful cryopreservation of intact human ovary with its vascular pedicle [42]. This was followed in 2008 by the announcement of Porcu and her colleagues on cryopreserved oocytes in cancer patients and the first ever birth of healthy twins after oocyte cryopreservation and bilateral ovariectomy [43].

1.8 Manipulation of the IVF Procedure and Technique to Solve Various Causes of Infertility

In 1983, the Monash IVF team achieved the first birth in a woman without ovaries by using donor eggs, creating artificial menstrual cycles, and a special hormonal formula for the first 10 weeks of pregnancy [44]. This came along with the first successful delivery following egg donation [45]. Still in

the same year, in vitro maturation (IVM) and fertilization of morphologically immature human oocytes in an IVF setup was developed and published [46]. However, it took 11 years until the first live birth as a result of IVM following transvaginal ultrasound-guided oocyte collection was reported. Further development of the technique was reported by Barnes et al. who achieved a blastocyst development from IVM oocyte that was fertilized by ICSI and underwent assisted hatching before transfer. This technique resulted in a healthy birth [47].

Utian et al. in 1985 were the first to describe a successful IVF surrogate pregnancy. This technique enables women without a functioning uterus to have a biological offspring. This includes women with an absent uterus as in Müllerian agenesis, Mayer–Rokitansky–Küster–Hauser syndrome, women posthysterectomy, and those with nonfunctioning uterus such as in Asherman syndrome.

The intended parents' embryo is transferred to the surrogate mother after synchronization of her uterine lining, as done with donor egg recipients. After the birth, the baby is given to its biological parents (Utian et al. 1985) [B].

Fertility preservation for cancer patients whose expected chemotherapy treatment (especially alkylating agents) might harm their ovarian reserve is gaining popularity. The improved prognosis and longevity with the increased concern of the quality of life made these treatments an integral part of the assessment and treatment of cancer patients. One concern with estrogen-sensitive breast cancer patients is the supranormal levels of estrogen during ovarian stimulation. New stimulation protocols utilizing letrozole (an aromatase inhibitor) were first reported by Oktay et al. (2005). The letrozole enables a full stimulation with lower estrogen levels and excellent results (Oktay et al. 2005) [C].

The role of assisted hatching is still controversial, and the indication for the procedure is not very clear. However, in 1985, Cohen and his colleagues reported in *The Lancet* about a birth after replacement of hatching blastocyst cryopreserved at the expanded blastocyst stage [48]. It was later on suggested to use this specific technique of assisted zona hatching to breach the zona pellucida and promote the natural process of hatching when the prognosis is poor [52].

1.9 Summary

The field of IVF was developed dramatically during the last 30 years, and what was considered to be a miracle has become a common practice around the world. The field is still being developed in multiple directions to find solutions for couples who are infertile, those wishing to conceive at an older age, and those needing to delay pregnancy for medical reasons.

We can expect further developments in the drug development, reduce the difficulties involved in stimulation of the ovaries moving into natural cycles, and obviously find a way to define the best embryo for replacement, which might give the patients the highest chance to become pregnant.

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Setting Up an ART Unit: Planning, Design and Organization

Contents

Setting Up an ART Unit: Planning, Design, and Construction

Jacques Cohen, Mina Alikani, Antonia Gilligan, and Tim Schimmel

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Learning Objectives

- To evaluate the requirements for staffing, of an ART laboratory
- To review facility design, engineering, equipment, and material requirements
- To discuss building materials and details of facility burn-in
- To prepare for procedures, maintenance planning, and risk management

2.1 Introduction

There are several alternatives for planning and operating an assisted reproductive technology (ART) laboratory; one setup may have little in common with another but may prove to be equally efficacious. Although this is important to remember as one ventures into establishing a new clinic, a poor design or plan may be hard to correct later. Facilities for ART range from a makeshift IVF laboratory with a minimum of equipment to a fully equipped laboratory specifically designed for ART and additional space dedicated to cryostorage, clinical care, and research. This chapter does not cover improvised laboratories, converted surgical areas or research facilities, or remote egg collection sites with transportation of gametes and embryos from other locations. Neither do we cover the application of simple, but ingenious, cost-reducing culture systems such as those described by Jonathan van Blerkom and coworkers in 2014 [1] or intravaginal culture (IVC) originally pioneered by Ranoux et al. (1988) [2] and redeveloped by INVO Bioscience more recently [3]. While such models can be successful, applications require egg retrieval and embryo transfer facilities. Whether these alternative approaches can achieve optimal results is still being investigated [1–4]. In vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), preimplantation genetic testing (PGT), and cryopreservation cannot be readily applied to alternatively cultured gametes and embryos [4, 5].

This chapter concerns the conventional all-inclusive laboratory that is adjacent or in immediate proximity to oocyte retrieval and embryo transfer facilities, with emphasis on the special problems of construction. For questions on culture system, culture medium and supplementation, selection methods and handling and processing of gametes and embryos including freezing and vitrification, the reader is referred to other relevant chapters in this textbook. The authors over the years have addressed the topics presented here in various formats and in other book chapters and papers. This chapter follows the format presented in Cohen et al. (2017) with additional data, advice, and information provided where appropriate [6].

2.2 Personnel and Experience

While the physical space dedicated to a purpose-built gamete and embryo culture facility deserves special consideration, it is the staff that will carry out the procedures and is essential to the success of the entire operation. Successful clinical practice in general, and ART in particular, is almost entirely dependent on the skill and experience level of medical and laboratory personnel. For the laboratory staff, enthusiasm is another key factor to success, especially because there are still few formal teaching and skills examination programs in place for a specialty in ART. Most clinical embryologists are trained via an apprenticeship program, but such institutions are rare and there are no internationally accepted guidelines. Good clinical outcomes require a close assessment of individual abilities, so laboratory staff, directors, and embryologists must consider their experience in the context of what will be required of them [7].

IVF clinics should participate in external proficiency testing programs and develop their own system of tracking staff performance in crucial clinical and laboratory procedures such as embryo transfer, ICSI, and embryo biopsy. Certain regulatory bodies such as the College of American Pathologists (CAP) and the American Board of Bioanalysis (ABB) in the USA and the Human Fertilisation and Embryology Authority (HFEA) in the UK provide standards of operation for laboratories, accreditation, or licensing for embryologists or laboratory directors. The HFEA in particular provides licensing for individual procedures such as ICSI and embryo biopsy. Licensing, however, is controversial because it does not guarantee skill or success and the licenses are not always recognized across borders from one country to another. Auditing and accreditation of laboratories, however, may have played a positive role in improving outcomes, because they encourage standardization and require a commitment to a quality management program that includes quality control and assurance. This improvement is clear in reported implantation rates in the USA [8].

Tradition also plays its role. For example, in some Asian countries, embryology directors are usually medical professionals. Thus, academic titles are often seen as being more important than practical experience. What then qualifies someone to be a laboratory director or an embryologist? The answer is not a simple one. In general, current licensing authorities including the ABB consider individuals trained in general pathology or reproductive medicine and holding an MD degree as well as individuals holding a PhD degree qualified to be laboratory directors if they meet some additional requirements. However, pathologists do not necessarily have experience in gamete and embryo cell culture, and some reproductive medicine specialists, such as urologists and immunologists, may have never worked with gametes and embryos. Although some medical doctors with laboratory experience do successfully direct laboratories, in many countries, the rules make it possible for anyone with a medical degree to direct a laboratory without having any proficiency in gamete and embryo handling! Once there are rules, even ones that make little practical sense, it may be hard to change them.

2.3 Staffing and Staff Training

There is considerable disagreement about what should be required experience for embryologists. Hands-on experience in all facets of clinical embryology is an absolute

requirement when starting a new program. Even highly experienced experimental embryologists and animal scientists should be directly supervised by experienced clinical embryologists. The period during which close supervision must continue depends on the types of skills required, the daily caseload, and time spent performing procedures. Clearly, performing 100 cases over a 1-year period is a very different circumstance than performing the same number over 6 weeks; the period of supervision then should be adjusted accordingly.

The optimal ratio of laboratory staff to the expected number of procedures is debatable, and unfortunately, economics play an all-too-important role here. However, with the incorporation of new technologies and treatment modalities in routine care, the complexity of IVF laboratory operations has increased substantially over the past decade, in turn requiring more careful consideration of staffing levels [9]. According to some calculations, while a "traditional" IVF cycle required roughly 9 personnel hours, a contemporary cycle can require up to 20 h for completion. Thus, the number of embryologists required for safe and efficient operation of the laboratory has also increased. Recently, based on a comprehensive analysis of laboratory tasks and their complexity level, an Interactive Personnel Calculator was introduced to help laboratory directors and administrators determine staffing needs [9]. This calculator is helpful, but it applies to one model of operation and would have to be modified to fit other models or special circumstances. For instance, a clinical approach that employs natural cycles or minimal stimulation yielding limited egg numbers is a very different model, compared to controlled ovarian stimulation with high oocyte yields. These differences notwithstanding, overall, it is safe to say that the ratio of laboratory staff to caseload should be high so that embryologists can not only safely perform procedures, but dedicate time to quality control and continued education and training in order to maintain the high standards required for success. The challenge of keeping these standards within national health systems or in the face of insurance mandates that must provide a wide range of services on a minimal budget is real, but should not be insurmountable. Needless to say, patients usually do not benefit from such constraints, as a comparison of results in different health service systems in Western countries would suggest. There are limitations to such comparisons, but live birth per embryo and cumulative data from fresh and cryopreservation cycles are considered objective assessments [10].

2.4 Embryologist Tasks

The job description for the embryologist ideally includes all embryology and andrology tasks. Embryologists are often involved in other important tasks as well, including communications with patients, follicular monitoring, genetic counseling, equipment maintenance, purchasing and receiving, and administration. However, it should be realized that these tasks may detract from their main responsibilities. First and foremost, the embryologist's duty should be to perform gamete and embryo handling and culture procedures. Secondly, but equally important, the embryologist should maintain quality control standards, both by performing routine checks and tests and by maintaining detailed logs of incidents, changes, unexpected events, and corrective measures. Across all these duties, the following seven positions can be clearly defined: director, supervisor, senior embryologist, embryologist, trainee, assistant, and technician. There may also be positions for others to do preimplantation genetic diagnosis, research, quality control supervision, or administrative work. Obviously, not all of these separate positions are necessary for smaller centers, and some tasks can be combined.

Although a seemingly unimportant detail, one of the most important jobs in the IVF laboratory at Bourn Hall Clinic in Cambridge, UK, during the first few years of operation in the 1980s was that of a professional witness and embryology assistant. This position was the brainchild of Jean Purdy, the third member of the team, whose work led to the birth of Louise Brown. The embryology assistant effectively enforced and oversaw the integrity of the chain of custody of gametes and embryos during handling, particularly when large numbers of patients were being treated simultaneously. The "witness" also ensured that embryologists performed only those procedures for which they were qualified. Interestingly, recent studies suggest that this crucial concept has not been universally and fully understood or adopted by IVF laboratories. In one group of laboratories [11], "limited and consequently virtually ineffective" witnessing processes were only abandoned in favor of a more robust witnessing program after implementation of a Failure Mode Effect and Analysis (FMEA) showed a high risk of error in gamete and embryo identification. The authors stated that, "Only after FMEA optimization has the witness embryologist been formally recognized as a committed role, specifically trained for witnessing shift work." Hopefully, the publication of this and other similar studies [12] that show the effectiveness of a witnessing system will encourage more laboratories to re-examine their practices and allocate adequate resources in ensuring safety and efficiency of all procedures performed by the laboratory.

2.5 Facility, Design, and Budget

As an important preface to this section, we must emphasize that setting up a new laboratory or renovating an existing facility must be a close collaborative effort between a team of experienced embryologists, medical doctors, and program administrators and a team of engineers, architects, and contractors. Without this collaboration, the ultimate success of such endeavors is put in jeopardy.

In the early days of IVF, some clinics were built in remote areas, based on the premise that environmental factors such as stress could affect the patient and thereby the outcome of treatment. Today's laboratories are commonly placed in city centers and large metropolitan areas in order to serve larger populations. It is clear that the choice of a laboratory site is

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of great importance for a new program. The recent development of better assays for determining the baseline quality of the environment facilitates site selection. There is now awareness that some buildings or building sites could be intrinsically harmful to cell tissue culture [13–15]. The direct effect of poor air quality and presence of volatile organic compounds (VOCs) on IVF outcomes has been demonstrated by recent studies of novel filtration systems and other counter measures [16, 17]. A recent consensus meeting on these specifics in relation to air quality was convened in Cairo in 2017, the results of which have been published [18]. A laboratory design should be based on the anticipated caseload and any subspecialty. Local building and practice permits must be assessed prior to engaging and completing a design.

There are four basic types of design:

1. Laboratories adjacent to clinical outpatient facilities that may be used part of the time. Other types of procedures may be performed in the procedure room or surgical suite (this may complicate air handling).
2. Clinics with inter-facility egg transport using portable warming chambers.
3. Fully integrated laboratories with adjacent clinical areas and procedure and transfer rooms dedicated exclusively to assisted reproduction.
4. Moveable temporary laboratories.

Before developing the basic design for a new laboratory, environmental factors must be considered. While air quality in modern laboratories can be controlled to a degree, it can never be fully protected from the exterior environment and adjoining building spaces. Designers should first determine if the building or the surrounding site is scheduled to undergo renovations, demolition, or major changes of any kind in the foreseeable future. City planning should also be reviewed. Past environmental data and trends should be reviewed, and accessibility of the IVF laboratory to maintenance staff should be ascertained. Activity related to construction can have a significant negative impact on the laboratory, so the possibility of future construction in the vicinity should be considered. Prevalent wind direction, industrial hazards presence, and general pollution levels (such as ozone measurements) should also be determined. Even when these factors are all deemed acceptable, basic air sampling and determination of VOC concentrations is necessary inside and outside the proposed building area. The outcome of these tests will determine which design requirements are needed to remove VOCs from the laboratory area. In most cases, an over-pressured laboratory (at least 0.10–0.20 inches of water) that uses a high number [7–15] of fresh air changes (FACH) per hour is the best solution, because it also provides for proper medical hygiene. The laboratory walls and ceiling should have the absolute minimum number of penetrations. This generally requires a solid ceiling, sealed lighting, and airtight utility connections. Contrary to many vendors' representations, commercial suspended ceilings using double-sided tape and clips are not ideal. Doors will require seals and sweeps and should be lockable. Ducts and equipment must be laid out in such a way that routine and emergency maintenance and repair work can be performed outside the laboratory with minimal disruption to the laboratory. Air handling must not use an open plenum design. In the ideal case, 100% outside air with chemical and physical filtration will be used with sealed supply and return ducts. Recommended conditions and more than 50 consensus points for air handling and facility operational aspects are discussed in detail in the Cairo consensus paper on the IVF laboratory environment [18].

While providing cleaner air, 100% outside air sourcing will maximize the life of a chemical filter and will provide a lower concentration of VOC in the IVF laboratory's air. In climates where temperatures routinely exceed 32 °C with >= 85% relative humidity, 100% outside air could result in an unacceptable level of humidity (>60%), which could allow mold growth. In these cases, the use of limited return air from the lab is acceptable. A 50% outside air system with 15–30 total air changes per hour does work well, and the relative humidity becomes very controllable. To place this in perspective, traditional medical operating room design in the USA calls for 10–15% outside air.

The air supply equipment may supplement outside air with re-circulated air, with processing to control the known levels of VOCs. On rare occasions, laboratories will require full-time air recirculation, while most may actually find the outside air to be perfectly clean at least most of the time. Outside air is often erroneously judged to be polluted without proper chemical analysis, while inside air is usually considered "cleaner" because it may "smell" better or seem less polluted due to the lack of a horizon [10]. However, in most laboratory locations, conditions are actually the reverse, and designers should not "follow their instincts" in these matters. Humidity must also be completely controlled according to climate and seasonal variation. The system must be capable of supplying the space with air with a temperature as high as 30–35 °C at less than 40% relative humidity. Air inlets and outlets should be carefully spaced to avoid drafts that can change local "spot" temperatures or expose certain equipment to relatively poor air or changes in air quality. Laminar flow hoods and micromanipulation workstations should not be located too close to air supply fixtures to avoid disruption of the sterile field and to minimize cooling on the microscope stage. Semi-enclosed workstations based on Class 2 cabinets or neonatal isolette incubators can be considered to optimize the work environment and bridge the gap between the incubator and the workstation. A detailed layout and assessment of all laboratory furniture and equipment is therefore essential prior to construction and has many other benefits.

Selection of an experienced and subspecialized (and flexible) architect and a mechanical engineer for the project is essential. Confirm what their past experience has been in building biologically clean rooms. The use of "environmentally friendly" or "green" products has been suggested by some designers. The reliance on "natural" products does not ensure a clean laboratory. In one case, wood casework with a green label was found to be a major source of formaldehyde. Floor coverings using recycled vinyl and rubber were

selected for their low environmental impact, without considering the significant release of trapped gasses by the material.

Supervision of the construction is also critical. Skilled tradesmen using past training and experience may not follow all of the architect's instructions. The general contractor and the builders must be briefed on why these novel construction techniques are being used. They must understand that the use of untested methods and products can compromise the project (and the payment of their fees!) Contractual agreement is recommended. They must be informed of preferred products used in IVF laboratory construction. A summary of this is provided elsewhere [18].

Just as the organization and flow of traffic in a world-class restaurant result in a special ambience where more than just the food is the attraction, appropriate modular placement of equipment ensures safety and comfort in the over-pressured IVF laboratory. Placement of stacks or small volume box or benchtop incubators, gamete handling areas (laminar flow units or isolettes), and micromanipulation stations should minimize distances that dishes and tubes need be moved. Ideally, an embryologist should be able to finish one complete procedure without moving more than 3 meters in any direction; not only is this efficient but also it minimizes accidents in a busy laboratory. Some newer laboratories apply modular design, where each technician remains in a small dedicated area. Design and implementation of a work area incorporating CO_2 and nitrogen supplies, a workstation, refrigerator, and incubators is feasible even without the embryologists having to walk between storage cabinets and equipment. Such a modular design can be duplicated multiple times within a larger air handling area allowing the handling of large numbers of gametes and embryos. For logistical reason, sperm preparation and cryopreservation may be placed in adjacent areas. The number of modules can easily be determined by the expected number of cases and procedure types, the average number of eggs collected, and the number of embryologists expected to work simultaneously. Each person should be provided with sufficient workspace to perform all procedures without delay. Additional areas can contain simple gamete handling stations or areas for concentrating incubators. Cryopreservation and storage facilities are often located in a separate space, although this is not strictly necessary; if separated, these areas should always be adjacent to the main laboratory. Storage spaces could be separated further using closets or rooms with negative pressure and air ventilation. Another separate laboratory or module may contain an area for culture medium preparation, sterilization, and water treatment; however, the need for such an area is diminishing now that commercial manufacturers provide all the basic needs of an IVF laboratory. Administration and other aspects of patient care should be performed in separate offices on a different air handling system from the main laboratories.

Last but not least, it is preferable to prepare semen in a separate laboratory altogether, adjacent to one or more collection rooms. The andrology laboratory should have ample space for microscopes, centrifuges, refrigerator, and safety cabinets. Proper separation of patient samples during processing is essential, and some elemental design features may be considered before the first procedures are carried out. Some thought should go into planning the semen collection area. This small room should be at the end of a hallway preferably with its own exit; it should be soundproofed, not too large, with a sink. Clear instructions of how to collect semen for ART should be provided in the room. The room should also be adjacent to the semen preparation laboratory, preferably with a double-door pass-through for samples. This pass-through should have a signaling device so the patient can inform the laboratory staff that the sample is ready; it also permits male patients to leave the area without having to carry a specimen container. Remote semen collection is a routine procedure in many clinics. It may be efficient and safe but requires that patients strictly follow clinic instructions (► www.sart.org).

2.6 Equipment and Storage

A detailed list of equipment should be prepared and checked against the planned location of each item; it can later be used as the basis of maintenance logs. It is important to consider the inclusion of crucial equipment and spare tools in the laboratory design, to allow for unexpected malfunction. Similarly, two or more spare incubators should not be seen as excessive; at least one spare follicle aspiration pump and micromanipulation station (equipped with laser) should also be included. There are many other instruments and pieces of equipment, the malfunction of which would jeopardize patient care, although some spares need not be kept on hand as manufacturers may have them available; however, such details need to be repeatedly checked as suppliers' stock continues to change. It may also be useful to team up with other programs or an embryology research laboratory locally so that a crucial piece of equipment can be borrowed in case of unexpected failure.

The number and type of incubators should be carefully considered [19]. The ratio of incubators to patient procedures depends on incubator size and capacity, and it varies considerably from program to program. It is clear that the number and type of incubator, routine maintenance, and cleaning as well as the length and number of incubator door openings may affect results. In principle, the number of cases per incubator should be kept to a minimum; we prefer a limit of four cases per large standard box incubator. The smaller box incubators should not handle more than two to three cases. Several new desktop incubators now have separate slots for handling one patient at a time. This is a good development. Several other incubators can be used for general purpose during micromanipulation and for other generic uses to limit further the number of incubator openings during extended culture. Strict guidelines must be implemented and adhered to when maintaining distinct spaces for separating culture dishes or tubes of different patients. Tracking of incubators and even shelves or compartments within each incubator is recommended so their performance can be evaluated on an ongoing basis. Separate compartments within a small-type

box incubator may be helpful and can be supplied by certain manufacturers. Servicing and cleaning of equipment such as incubators may have to be done when the laboratory is not performing procedures. Placement of incubators and other pieces of equipment on large castors may be helpful in programs where downtime is rare. Pieces of equipment can then be serviced outside the laboratory. New incubators and pieces of equipment that come in contact with gametes and embryos must be "burned-in" or "off-gassed." Protocols vary per equipment type and manufacturer [18].

When there are several options available to the laboratory designer, supply and evacuation routes should be planned in advance. One of the most susceptible aspects of ART is cryopreservation and cryostorage [20]. In case of an emergency such as fire or power failure, it may be necessary to relocate the liquid nitrogen-filled dewars without using an elevator or to relocate the frozen samples using a temporary container. This may seem like an extreme measure, especially in the larger laboratories that stockpile thousands of cryostored samples, but plans should be made and risk management is essential given the possibility of catastrophic failures, which have been reported [20]. It may be possible to keep a separate storage space or room near the building exit, where long-term-storage samples, which usually provide the bulk of the storage, can be kept; this would require daily monitoring and alarming of a facility that is not part of the laboratory. Liquid nitrogen tank alarms with remote notification capability should be installed on all dewars holding gametes and embryos. A minimum of two monitoring systems is highly recommended.

The route of delivery of liquid nitrogen and gas cylinders must be relatively short, without stairways between the laboratory and the delivery truck, and should be sensibly planned in advance. Note that the flooring of this route is usually destroyed within months because of liquid nitrogen spills and wear caused by delivery containers, so the possibility of an alternative delivery corridor or installation of special LN2-resistant flooring should be considered for these units.

Liquid nitrogen containers and medical gas cylinders are preferentially placed immediately adjacent to the laboratory in a closet or small, ventilated room with outside access. Pipes and tubes enter the laboratory from this room, and cylinders can be delivered to this room without compromising the laboratory area in any way. Providing liquid nitrogen and even liquid oxygen vapor to triple gas incubators is nowadays a preferred option since vapor is cleaner and more affordable than compressed gas. This allows liquid nitrogen vapor to be pumped into the cryopreservation laboratory using a manifold system and minimal piping. Lines should be properly installed and insulated to ensure that they do not leak or allow condensation and conserve energy at the same time. Medical gasses can be directed into the laboratory using prewashed vinyl/Teflon-lined tubing such as fluorinated ethylene propylene or FEP, which has high humidity, temperature, and UV radiation stability. Lines should be properly marked every meter indicating the incubators supplied in order to facilitate later maintenance. Alternatively, solid manifolds made from stainless steel with suitable compression fittings can be used. Soldered or brazed copper lines used in domestic plumbing applications should be avoided wherever possible; copper lining can be used but should be cleaned and purged for a prolonged period prior to use in the laboratory. Copper line connections should not be soldered as this could cause continuous contamination. This recommendation may conflict with existing building codes, but non-contaminating alternatives can be found. A number of spare lines hidden behind walls and ceilings should be installed as well, in case of later renovation or facility expansion.

Large programs should consider the use of exterior bulk tanks for carbon dioxide and liquid nitrogen. This removes the issues of tanks for incubators or cryopreservation. These tanks are located where delivery trucks can hook onto and deliver directly to the tank. Pressurized gas lines or cryogenic lines then run the carbon dioxide or liquid nitrogen to the IVF laboratory for use.

Placement of bulky and difficult pieces of equipment should be considered when designing doorways and electrical panels. Architects should be fully informed of all equipment specifications to avoid the truly classic door-width mistake. Emergency generators should always be installed, even where power supplies are usually reliable. The requirements can be determined by an electrical engineer. Thankfully, these units can be well removed from the laboratory but must be placed in well-ventilated areas that are not prone to flooding. Additional battery "uninterruptible power systems" (UPS) may be considered but can be of limited capability or expensive when the requirement is to run the entire clinic for 1 day or longer. Buildings should also be checked for placement of the main power inlets and distribution centers, especially because sharing power lines with other departments or companies may not be advisable. Circuit breakers should be easily accessible to laboratory staff or building maintenance staff. General knowledge of mechanical and electrical engineering of the building and the laboratories is always advantageous. Leaving all the building mechanics and facilities to other individuals can be counterproductive. Laboratory staff need to be involved with facilities management and be updated with construction decisions in and outside the building in a timely manner.

Ample storage spaces should always be planned for IVF laboratories. In the absence of dedicated storage space, laboratory space ends up being used instead, filling all cabinets and negating any advantages of the original design. The dedicated storage area should be used to stock all materials in sufficient quantity to maintain a steady supply. A further reason to include storage areas in laboratory design, sufficient on its own to justify the space, is that new supplies, including sterile disposable items, release multiple compounds for prolonged periods. This "outgassing" has been determined to be a major cause of air pollution in a number of laboratories in which supplies were stored inside the lab. Separate storage space therefore provides the best chance of good air quality, especially when it is supplied by a separate air handling system.

It should be large enough to handle bulky items as well as mobile shelving for boxes. One should be careful to avoid the natural inclination to save extra trips by bringing too many items into the laboratory, or the gains made by careful design may be lost. As a possible makeshift solution, storage cabinetry in the laboratory can be designed with separate negative pressure air handling in order to minimize release of VOCs from off-gassing package materials.

2.7 Microscopes and Visualization of Cells

Though dissecting microscopes are crucial for the general handling of gametes and embryos, inverted microscopes are key during morphology assessment and micromanipulation. Proper visualization of embryos is key to successful embryo selection for transfer or freezing; if the equipment excels, visualization can be done quickly and closely. Even so, appropriately detailed assessment is still dependent on the use of an oil overlay system to prevent damage by prolonged exposure. Each workstation and microscope should be equipped with a camera as well as a monitor. Still photos can be placed in the patient file or uploaded electronically in the database, and video footage like time-lapse permits speedy review of embryonic features with colleagues after the gametes are safely returned to the incubator; this is also helpful for training of new embryologists. Interference optics such as Hoffman and Nomarski are preferable because they permit the best measure of detail and depth. Novel visualization of internal elements such as spindles using polarized microscopy requires additional equipment but can be incorporated into routine operation [21].

Development of new time-lapse microscopy technologies has made continuous and uninterrupted monitoring of embryo development a reality. This is not just an invaluable teaching and learning tool, but also provides a permanent record for patient care. However, equipment costs are high and, for many laboratories, prohibitive. Equipment for time-lapse technology can be sizable and may require separate consideration in terms of lab design, bench space, and CPU placement.

2.8 Construction, Renovation, and Building Materials

Construction and renovation can introduce a variety of compounds into the environment of the ART laboratory, either temporarily or permanently. Either can have major adverse effects on the outcome of operations [13, 14, 16–18]. The impact of the exterior environment on IVF success has been demonstrated. Pollutants can have a significant negative effect on success in an IVF laboratory [13, 22]. These effects can range from delayed or abnormal embryonic development, reduced or failed fertilization, and reduced implantation rates to pregnancy loss and failure of a treatment cycle. Many of the damaging materials are organic chemicals that are released or outgassed by paint, adhesives from flooring, cabinets, and general building materials, as well as from laboratory equipment and procedures. It is important to realize that the actual construction phase of the laboratory can cause permanent effects on clinical outcomes. Furthermore, any subsequent renovation activity in adjacent areas may also have an effect. Neighboring tenants can be informed of the sensitivity of gametes and embryos in culture. At the very least, changes undertaken in adjacent areas should be supervised by IVF laboratory personnel to minimize potential damage. However, new construction immediately outside the building is considerably more problematic. City works such as street construction are very hard to predict and nearly impossible to control. A good relationship with the neighbors should be maintained and a working relationship with building owners and city planners should be established so that the IVF laboratory is kept informed of upcoming changes.

2.9 Preparation for Construction

For the construction of a new laboratory or if changes are to be made to areas adjacent to the IVF facility, the following guidelines should be followed [6, 18]. First, the area to be demolished and reconstructed needs to be physically isolated from the IVF laboratory (if this is not the new IVF laboratory itself). The degree of isolation should be equivalent to an asbestos or lead abatement project. The isolation should be done through (1) physical barriers, consisting of polysheeting supported by studding where needed; (2) limited access to the construction area and the use of an access passageway with two doors in series; (3) removal of all construction waste via an exterior opening or proper containment of waste before using an interior exit; (4) negative air pressure in the construction area, exhausting to the exterior, far removed from the laboratory's air intake, and properly located with regard to the prevailing winds and exterior airflow; (5) extra interior fans during any painting or the use of adhesives to maximize removal of noxious fumes; and (6) compiling and logging of Safety Data Sheets (SDS) for all paints, solvents, and adhesives in use.

Follow-up investigations with manufacturers and their representatives may be helpful because specifications of equipment may be changed without notice. The negative pressurization of the laboratory space requires continuous visual confirmation via a ball and tube pressure indicator or simply paper strips. Periodic sampling for particulates, aldehydes, and organics could be done outside the demolition and construction site, provided this is economically feasible. Alternatively, tracer gas studies can be done to verify containment. The general contractor of the demolition and construction should be briefed in detail on the need to protect the IVF facility and techniques to accomplish this. When possible, the actual members of the construction crew themselves should be selected and briefed in detail. Large filter

units using filter pellets of carbon and permanganate can be placed strategically. Uptake of organics can be assayed, but the frequency of routine filter changes should be increased during periods of construction activity.

2.10 Selection of Building Materials

Many materials release significant amounts of VOCs, and a typical list includes paints, adhesives, glues, sealants, and caulking materials, which release alkanes, aromatics, alcohols, aldehydes, ketones, and other classes of organic materials. This section outlines steps to be taken in order to reduce these outgassing chemicals. Any and all interior painting throughout the facility should only be done on prepared surfaces with water-based paint formulated for low VOC potential. During any painting, auxiliary ventilation should be provided using large industrial construction fans, with exhaust vented to the exterior. Paints that can significantly influence air quality should be emission tested (some suppliers already have these test results available). Safety Data Sheets are generally available for construction materials. Suppliers should be encouraged to conduct product testing for the emission potential. The variety of materials and applications complicates the testing process, but several procedures have been developed to identify and quantify the compounds released by building materials and furnishings. Interior paints must be water-based, low-volatile paints with acrylic, vinyl acrylic, alkyd, or acrylic latex polymers. Paints meeting this specification can also contain certain inorganic materials. Paints with low volatiles may still contain low concentrations of certain organics. No interior paint should contain formaldehyde, acetaldehyde, isocyanates, reactive amines, phenols, and other water-soluble volatile organics.

Adhesive glues, sealants, and caulking materials present some of the same problems as paints. None of these materials used in the interior should contain formaldehyde, benzaldehyde, phenol, and like substances. Although water-based versions of these are generally not available, their composition varies widely. Silicone materials are preferred whenever possible, particularly for sealants and caulking work. A complete list of guidelines for material application during construction of a tissue culture laboratory is available elsewhere [23].

2.11 "Burning In" of the Finished Facility and Outgassing of New Equipment

New IVF laboratories and new facilities around existing laboratories are occasionally affected by complaints of occupants who experience discomfort from the chemicals released by new construction and furnishings. Some materials may outgas for years. Effects on gametes and embryos must be avoided by using nontoxic materials and filtration [6, 18, 22, 23]. The ambient levels of many of these materials can be reduced by "burning in" the facility. A typical burn-in consists of increasing the temperature of the new area by 10–15 °C, if possible, and increasing the ventilation rate; even higher temperatures are acceptable. The combination of elevated temperature and higher air exchange aids in the removal of the volatile organics. Upon completion of the construction, the air handling system should be properly configured for the burn-in of the newly constructed area. As previously stated, the system must be capable of supplying the space with air with a temperature of 30–35 °C, at less than 40% relative humidity. The burn-in period can range from 10 to 28 days, and the IVF laboratory should be kept closed during this time. If these temperatures cannot be reached by the base system, use auxiliary electrical heating to reach the minimum temperature. During burn-in, all lighting and some auxiliary equipment should be turned on and left running continuously. Naturally, ventilation is critical if redistribution of irritants is to be avoided; the whole purpose is to purge the air repeatedly. Auxiliary equipment should of course be monitored during the burn-in.

The same burn-in principle applies to newly purchased incubators or other laboratory equipment. Removal of volatile organics is especially important in the critical microenvironment of the incubator. Whenever possible, it is advantageous to purchase incubators months in advance of their intended initial use and to operate them at an elevated temperature in a clean, protected location, preferably not an IVF facility. An existing embryology laboratory is not an appropriate space for burn-in of a new incubator. Most of the equipment available for use in an ART laboratory has not been designed or manufactured to be VOC-free. Special attention must be invested in new laboratory equipment to eliminate or reduce VOC levels as much as possible before first use. Manufacturers should be encouraged to design IVF-specific equipment and choose parts with low emission potential. Most manufacturers do not address the issues of VOC outgassing in product manuals even if the equipment has been expressly designed for the IVF field. Unpacking, cleaning, and operating equipment prior to final installation in a lab for outgassing the "new car smell" is always recommended.

Incubators should be unpacked, inspected, cleaned, outgassed, operated, re-cleaned, calibrated, and tested well in advance. The process can take several months but is an essential task rewarded with the most suitable culture system the selected incubator model can provide. When possible, operating incubators at elevated temperatures above the typical culture temperature will hasten the release or burn-off of VOCs. Extended operation between 40 and 45 °C works well to burn off VOCs if this is within the manufacturer's recommended temperature range. Incubator model VOC loads can vary greatly. Accurate VOC testing may be expensive and time-consuming but is recommended to test a specific incubator model to determine the

new unit's typical VOC characteristics and how much time outgassing may require.

Handheld VOC testing devices are available and can be used to help monitor the decline of total VOCs but cannot match the level of accuracy of an environmental organic chemist's testing. Handheld VOC meter technology generally is not sensitive enough to monitor low molecular weight classes of VOCs, but they are reasonably affordable and easily used and can sometimes provide confirmation of some VOC reduction.

New incubators are generally tested with a mouse embryo assay (MEA) replicating a culture system as part of a new incubator commissioning process. Most laboratories today use some variation of an oil culture system. The oil can serve as an excellent filter against potential VOCs, but may not protect a culture system from the full range of VOC exposure. In particular, low molecular weight compounds such as aldehydes may affect the culture system negatively. Incubator MEA commissioning should include both an oil and an open exposed media test to help evaluate the incubator preparation procedures. The dual oil/no oil approach works well for humidified incubators, but may not be applicable if a dry non-humidified culture system is used. Most non-humidified dry culture systems are designed to recirculate chamber air and incorporate a VOC filtration strategy. Open culture generally cannot be used with non-humidified incubators. The manufacturer's recommendations should be followed. Non-humidified incubators may require extended off-gassing and should be tested prior to use to confirm they do not have a VOC issue. Chemical VOC filters should be replaced after burn-off prior to any MEA testing. The humidification of a benchtop incubator was tested in a recent randomized clinical trial [24]. It was shown that pregnancy was significantly increased when the incubator was humidified, even though the manufacturer did not recommend it. However, this work needs further confirmation.

Laminar flow hoods and isolettes are also an important potential VOC source that should not be overlooked. They should be given ample time to operate and outgas as they can contribute to a lab's VOC contamination load. HEPA and chemical filters should be selected for low VOC manufacturing traits and also may require off-gassing. Care must be taken when outgassing laminar flow hoods and isolettes as they require a HEPA-filtered environment or replacement of their filters when transferred to an IVF lab.

After the burn-in is complete, a commissioning of the IVF suite should be conducted to verify that the laboratory meets the design specifications. The ventilation and isolation of the laboratory should be verified by a series of tests using basic airflow measurements and tracer gas studies. The particulate levels should be determined to verify that the high-efficiency particulate air (HEPA) system is functional. Particulate sampling can be performed using USA Federal Standard 209E. Microbial sampling for aerobic bacteria and fungi is often done in new facilities using an Andersen sampler followed by microbiological culturing and identification. The levels of VOC contamination should be determined. Possible methods are included in the US Environmental Protection Agency (EPA) protocols using gas chromatography/mass spectroscopy (GC/MS) and high-performance liquid chromatography sensitive at the microgram per cubic meter level [25–30].

2.12 Maintenance Planning and Sterilization

Even the best systems and designs will eventually fail unless they are carefully maintained. The heating, ventilation, and air conditioning (HVAC) will require filter changes, coil cleaning, replacement of drive belts, and chemical purification media. The most prevalent failure concerns the initial particulate filter. These are inexpensive filters designed to keep out large dust particles, plant debris, insects, etc. If such filters are not replaced promptly and regularly, they will fail, allowing the HVAC unit to become contaminated. The HEPA filters and chemical media also require inspection and periodic replacement. Maintenance staff should report their findings to the IVF laboratory.

The ART facility must have cleaning rooms for surgical instruments. Ongoing use of an autoclave is not a problem as long as the released steam is rapidly exhausted to the outside. This keeps the relative humidity in the facility to controllable limits. Autoclaves should not be placed on the IVF laboratory's HVAC system, but rather in a room that is built using tight construction and is exhausted directly outside of the building. The use of cold sterilizing agents is not advised. Aldehydes such as glutaraldehyde and *ortho*-phthalaldehyde from the autoclave can be transported inside the IVF laboratory.

2.13 Insurance Requirements

Assisted reproductive technologies have become common practice worldwide and are regulated by a combination of legislation, regulations, or peer-generated practice standards [18]. The rapid evolution and progress of ART reveal new legal issues that require consideration. Even the patients themselves are changing, as it becomes more acceptable for single women and same-sex partners to seek and receive treatment. Donation of gametes, embryos, and gamete components, enforcement of age limits for treatment, selective fetal reduction, preimplantation genetic diagnosis, surrogacy, and many other practices in ART present practitioners and the society at large with challenges, which are often defined by social norms, religion, and law and are specific to each country.

Furthermore, financial and emotional stresses often burden patients seeking treatment in countries where medicine is not socialized and infertility treatment is not covered by insurance. This translates into an increasing number of ART lawsuits related to failed treatments in spite of generally improved

success rates [8]. Laboratory personnel and the laboratory owner should therefore obtain an insurance policy of sufficiently high level and quality commencing prior to the first day of operations. Litigation-prone issues need special consideration and include (I) cancellation of a treatment cycle prior to egg retrieval, (II) failure to become pregnant, (III) patient identification errors [31], and (IV) cryostorage mishaps [20]. Adverse events can occur even if experienced practitioners consider themselves at low risk of exposure. Prior to engaging in the practice of ART, protocols must be established to identify potential problem areas and establish countermeasures.

2.14 Conclusions

It may be surprising how many professionals continue to pursue the establishment of new ART clinics at a time when competition is fierce, financial benefits are small, and existing ART services may appear to be approaching saturation in many areas and countries. Appearances can be misleading, however, and ART centers of excellence that deserve the trust and confidence of patients and serve as models for other practices are always needed.

This chapter provides some guidance for those who aspire to establish such outstanding, well-thought-out, and planned ART practices. Although it cannot safeguard practitioners against adverse events, it introduces concepts in proper design, construction, and operation of ART facilities that are of fundamental importance to treatment success; these guidelines have been painstakingly compiled through decades of practical experience and research. The approach is best adopted as a whole rather than dissected into its components and adopted in part or selectively. Resisting the urge to cut corners in the wrong places avoids future headaches and positions an ART program on the path to success.

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Building the Laboratory

Dean E. Morbeck and Marlena Duke

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Learning Objectives

- To understand the key differences in laboratory design for different size clinics.
- Describe the essential elements of the plumbing, electrical, and alarm systems.
- Process for validation before patient care.

3

3.1 Introduction

As new in vitro fertilization (IVF) laboratories continue to appear in private practice settings, it is paramount that the reproductive endocrinologist and laboratory director are well-versed in laboratory design. Unlike large institutions, where specialized engineers and contractors are available to design and build clean room-quality operating rooms and laboratories, professionals in the assisted reproductive technologies (ART) must navigate a specialty for which they have little training or experience.

Unfortunately, there are limited resources and references available to ART professionals when setting out to build a new laboratory. Standards for IVF laboratories published by professional societies and accrediting agencies are mostly lacking, and those that exist are superficial at best. Both ESHRE and ASRM provide minimal guidelines for laboratory space and design [1, 2]. This leaves the individual responsible for the design and supervision of building the laboratory to rely on other sources, particularly standards that exist for operating rooms and clean rooms. These two types of rooms represent the range of quality one should aspire for their facility: the operating room at a minimum and the class 10 or class 100 clean room as the maximum. Even within these types of facilities, there are aspects of design and management that are unique to an IVF laboratory.

This chapter focuses on the critical aspects of building a laboratory in a private practice setting as part of an ART clinic. The details presented here will delve into issues of laboratory design and construction, with the notable exception of the heating, ventilation, and air conditioning (HVAC) system. The goal of the well-designed IVF laboratory is an environment that fosters both good working conditions and air quality compatible with excellent IVF outcomes. Air quality is a function of HVAC design, room design, choice of building materials, and activities conducted therein and adjacent to the laboratory. Air quality management deserves its own chapter and merits emphasizing the importance of hiring a design-build engineer who specializes in HVAC requirements for clean room facilities. There are other subtle aspects of laboratory design and build that are not covered in this chapter, and the reader is encouraged to review other literature on the subject [3–10].

3.2 Laboratory Layout

3.2.1 General Considerations

Careful thought should go into the design and organization of the laboratory in order to minimize risks to gametes/embryos while maximizing embryologist comfort, efficiency, and use of space. Workstations should be organized and positioned in a manner that allows one embryologist to complete one procedure without moving more than 3 meters in any direction [6]. Though ideal, this design is often constrained by the size of the space available and the number of IVF cycles performed in a given time period.

Workload is an important consideration for the design of the laboratory and is influenced not only by how many IVF cycles are done in a year but whether the program batches their cycles and the amount of preimplantation genetic testing (PGT) is performed. The workload as a function of cycles per day dictates the number of dissecting microscopes and inverted microscopes with micromanipulation needed as well as the number of incubators required. For the latter, a standard that most embryologists adhere to is no more than four patients per standard CO_2 or triple-gas incubator, though this limit is becoming a function of incubator design, with new benchtop models and time-lapse incubators providing dedicated chambers to each patient. The layout of the laboratory is often constrained by space limitations and affected by personal preference.

Ideally, embryology laboratories are divided into two or more rooms to separate workspaces. Usually, the main embryology procedures are performed in one room adjacent to both the operating room and possibly other support rooms for semen preparations, micromanipulation, embryo biopsy and tubing, cryopreservation, media preparation, or supply cleaning. This layout facilitates the desired air pressure differential by enabling the main embryology room to have positive airflow to the adjacent areas. A commonly overlooked component of IVF laboratories is adequate storage space. Many embryologists prefer to purchase large lots of key supplies, which allows simplified tracking of exposure of patient tissues to contact materials. Additionally, many laboratory supplies are plastics, which release volatile organic compounds (VOCs) that may adversely affect embryo development. Therefore, embryologists prefer to allow the plasticware to off-gas in a separate storage area before use. Thus, it is important to have dedicated space for bulk storage and then bring only small amounts of the supplies into a support room prior to introducing them into the embryology laboratory proper.

The following are design approaches to two different size IVF laboratories.

3.2.2 Small IVF Center (<500 Cycles/Year)

Small IVF laboratories are by far the most common, since most IVF centers in the USA have one or two physicians who perform 100–150 retrievals each year. While this number is typical, some solo practitioners perform as many as 300 or more cycles per year. These small ART programs often batch patients into four to six "series" per year, with a series lasting for 1–4 weeks. Most large programs are open nearly year-round, so programs that batch 100 patients in four 2-week series are similar in workload to a practice that performs 600 cycles per year. As you might expect, limiting incubator

utilization to four patients per unit will require more incubators per annual patient volume in the batched style of practice relative to the program that performs cycles year-round.

Design of the laboratory layout depends on the amount of equipment and the number of workstations. The smallest IVF program requires, at a minimum, the same amount of equipment as a program that performs <300 oocyte retrievals on a year-round basis. All IVF laboratories should have at least two incubators, though with smaller benchtop incubators this can easily be increased to three, where one large, traditional upright incubator is used for equilibrating dishes and benchtop incubators are for embryo culture. As the IVF volume grows, the laboratory can add another full-sized incubator or purchase more benchtop incubators to function as working incubators.

In addition to two incubators, the small IVF laboratory requires one embryology and one andrology workstation. The embryology workstation consists of a dissecting microscope and an inverted microscope with micromanipulators. The most common configuration of these two microscopes is the dissecting microscope in a laminar flow hood and the inverted microscope on a vibration-free table. While this is the most common setup, there are many variations including placing the dissecting microscope in an isolette, placing both dissecting and inverted microscopes in a hood or omitting the laminar flow hood from the plans entirely. The choice of a laminar flow hood is based on air quality and experience: good HEPA filtration in the HVAC usually circumvents the need for hoods, whereas a certified clean room design makes them unnecessary [5].

A typical design for the small laboratory consists of two to three rooms: one for embryology, one for biopsy/PGT, and the other for cryostorage and preparation of sperm. The equipment in the second room includes a centrifuge, a compound microscope with 20x and 40x objectives, cryopreservation instruments, liquid nitrogen storage dewars, and a refrigerator. If there is a separate andrology laboratory that performs sperm preparations for IVF, this room can be used for setting up dishes for the embryology laboratory. Otherwise, media and dishes from the second laboratory should be carried to the embryology laboratory in anticipation of culture preparation. Air pressure should go from the embryology laboratory to the andrology laboratory to the hallway. Similarly, air pressure should move from the embryology laboratory to the operating room to the hallway.

There are two common designs for access between the embryology laboratory and the operating room (OR). For programs with enough space, a full-sized door connects the two rooms, and this allows a circulating OR technician to carry follicular aspirates directly into the laboratory during the retrieval procedure and an embryologist to carry the embryo transfer catheter directly to the physician for the transfer procedure. Having a door also allows the use of portable IVF workstations. Alternatively, programs with limited space often have only a pass-through window to transport tubes from the OR through to the laboratory during the oocyte retrieval. This also requires a hand-off of the transfer catheter through the window, but otherwise is advantageous in that it limits the airflow between the rooms and maximizes space utilization. For small programs, this works well except in the situation where the catheter is after-loaded by the embryologist.

3.2.3 Large IVF Center (>500 Cycles/Year)

Although small IVF laboratories are more common overall, the number of laboratories that perform >500 cycles/year as well as laboratories that perform >1000 cycles/year increases as the demand for fertility services continues to grow. These large laboratories often not only serve the physicians managing the practice but affiliate physician offices as well. Because of the high volume of cases, large IVF centers are usually open year-round or only closed for one or two brief maintenance periods per year.

In order to design a laboratory with efficient and comfortable workflow, the first step is to determine the estimated number of cycles the laboratory is expected to perform in a given time period. The estimated caseload will determine the required size of the laboratory proper and the quantity of adjacent support rooms. The amount of workstations, microscopes, micromanipulators, and other equipment is a factor of caseload as well. Large IVF laboratories with numerous physicians must develop a system to regulate the maximum number of cases per day or per week so that incubators are not overfilled 1 week and almost barren the next.

A detailed layout of the equipment must be undertaken before finalizing any constructions plans. Workflow must be carefully considered so long walks are minimized between workstations (hoods or isolettes) and incubators. Separate workstations are critical to avoid collisions of embryologists carrying dishes. Unfortunately, space may be limited when IVF centers are located in a city center. Ideally, extra space can be incorporated in original construction plans to allow for later expansion and adding workstations and incubators without interrupting workflow. Laboratory doors should always be wide enough to allow for new equipment delivery as needed.

The large IVF laboratory should consist of a main embryology room containing the bulk of the incubators, workstations, and micromanipulators. There is no magic number of incubators required for a specific caseload. A center performing 500 cases per year may find half a dozen incubators adequate, while 2 dozen incubators or more may be required to accommodate a center juggling 2000 cases annually. Patients reactive for infectious diseases may require isolation of their gametes during culture, which necessitates more incubators. Periodic cleaning of incubators requires extra incubators to handle the load of those temporarily out of service. It is most important to factor in the frequency of incubator door openings, rather than incubator capacity, because limiting incubator openings will better maintain the desirable culture conditions of temperature and pH for all patient material contained therein. For the large laboratory, the number of workstations, such as a laminar flow hood or isolette fitted with a dissecting microscope, is simple to calculate. To maintain an effi-

cient pace without delay while waiting for a place to perform procedures, approximately 1 workstation is needed for every 200–300 cases. Placing a customized plexiglass divider in a traditional large laminar flow hood allows the creation of two workstations each with its own microscope to maximize the use of space.

Support rooms adjacent to the main embryology laboratory are convenient to separate work areas and allow for sequential step-downs in air pressure. Some laboratories have a dedicated micromanipulation room containing a workstation, inverted microscope with manipulators, and a working incubator to utilize during procedures. The large laboratory will need at least two micromanipulation stations to accommodate high utilization of ICSI and PGT procedures. Semen processing is almost always conducted in a separate room. The room containing one or two semen processing workstations should be positioned near the collection room(s). It is highly recommended to have two collection rooms if space allows. A separate cryopreservation room is desirable for the cryopreservation equipment, storage dewars, workstation, and working incubator. Often the long-term frozen material which can fill dozens of dewars is maintained in a secondary secure room with easy street access in case of an emergency or natural disaster. Regardless of the amount of support rooms designed around the main embryology laboratory, the OR must be directly connected to embryology by way of a door or pass-through or both depending on the preference of the laboratory and physician team.

3.3 Room Construction

The goal for the physical construction of the IVF laboratory is to build an environment that is free of factors that might directly affect gamete and/or embryo viability. Thus, it is important to carefully consider all construction materials used to build the laboratory. Second to the HVAC design and build, this section is one that requires close attention by the laboratory director to ensure the building contractor understands the critical nature of the IVF laboratory.

A primary goal during construction of the physical structure of the laboratory is to avoid the use of materials that will release embryotoxic gasses for an extended period following completion of the construction. As will be described later, the laboratory should be given time after the completion of building to off-gas and be validated prior to clinical use. Because different materials off-gas at different rates, it is critical to choose building materials carefully and to verify that the selected materials are used.

Choice of flooring is standard for operating rooms and clean rooms: seamless sheet vinyl that has an integral base flashed at least 4 inches up the wall. Where pieces of flooring meet, the seam is sealed. This type of flooring seals odors out from materials used in the subfloor and the base of the walls. Compared to tile flooring, it is easily cleaned and does not allow water to seep under the walls. Flooring should also be flashed onto the base of cabinets in the laboratory, again to prevent a breeding ground for microbes. Steel is the material of choice for cabinetry in the IVF laboratory.

Walls should be low-odor, washable, and non-shedding. Choices for material for walls include plaster and plastic wallboard. While the latter does not have to be painted, only water-based paints formulated for low-VOC potential should be used. Epoxy paints can also be used, but they may require longer off-gassing periods. Similar to the flooring, the walls should be sealed. While most sealants are likely to off-gas VOCs, sealants made of silicone materials are a better choice if available.

Prior to the introduction of clean room technology, solid plaster ceilings, with minimal openings for fixtures, were considered acceptable for IVF laboratories. While this approach provides an airtight, non-shedding surface, it also presents challenges. Optimal HVAC design does not require access above the IVF laboratory; however, it is not always possible to avoid placement of ductwork or other mechanical systems above the IVF laboratory. Clean rooms typically use tiled ceilings with strip sealing to make them airtight. Unlike standard ceiling tiles used in commercial and residential applications, clean room ceiling tiles are non-shedding and can be sealed.

In addition to supply ducts, usually as terminal HEPA filters, openings in the ceilings should be limited to canned lights. Short-wavelength light, like that emitted from fluorescent light bulbs (470–480 nm), has been shown to be detrimental to cell culture and embryo culture [11–14], though it is not clear if this applies to all lighting in the room or just light emitted from the microscopes [13]. Most IVF laboratories use either incandescent light bulbs or cover fluorescent lights with UV sleeve protectors [6]. All lights and utility connections should be sealed and airtight.

3.4 Laboratory Support Systems

More than any other laboratory, the embryology laboratory requires an extensive support network of plumbing, electrical, and alarm systems. These systems, similar to the many configurations available for laboratory design, are scalable and available in both low- and high-tech options. While some regulatory agencies such as the College of American Pathologists (CAP) require certain critical equipment to be alarmed, no standards exist for these systems.

3.4.1 Plumbing

One of the biggest advantages of constructing a new IVF laboratory is the ability to place gas outlets strategically throughout the laboratory for incubators and gassing devices. Small laboratories may find this approach cost-prohibitive, but the advantages outweigh the additional cost. While covers can be used for tanks in the laboratory proper, the tanks are still dirty and present a physical hazard. With the clear benefit of reduced oxygen for embryo development in vitro [15], CO_2

and N_2 lines at a minimum should be placed at each location that will hold an incubator. With the advent of incubators that use premixed gas, an "air" line should also be placed at each port [16]. This allows the use of trigas (89% N_2/6% CO_2/5% O_2) for gassing dishes as well as for incubating units that require premixed gas.

Placement of the gas tank room can also be optimized with new construction, thus allowing easy access for the supply company to change out tanks while not disturbing operations in the laboratory or clinic. The tank room requires an oxygen sensor/alarm and automatic switching stainless steel manifolds. The number of couplings per manifold per gas will depend on the number of incubators in use but typically is two tanks per side. While stainless steel lines are preferred, copper lines can be used as long as the lines are not soldered [8].

Since reduced O_2 incubators use nitrogen to displace atmospheric O_2, a large supply of nitrogen is needed. Nitrogen gas can be provided in at least three ways: via compressed N_2 in cylinders, from a nitrogen generator that fills a compressed tank, or from the vapor phase of a liquid nitrogen tank. Because of the volume required, nitrogen cylinders should only be used when only two incubators are in use; otherwise, tank change-outs will be a daily chore. A nitrogen generator is an effective alternative, but the units are loud, relatively large, and require maintenance. Using the nitrogen vapor of liquid nitrogen tanks plus compressed nitrogen cylinders as backup on a manifold provides the most cost-effective and practical solution. Nitrogen tanks can also serve as an emergency backup for topping off Ln2 storage dewars. Although low-pressure LN2 tanks (psi < 30) may be required for some controlled rate freezers, it is essential to use high-pressure LN2 tanks (psi >150) when supplying N_2 for triple-gas incubators to maintain a constant pressure in the line for several incubators at once. Failure to supply adequate nitrogen will cause both the O_2 and the CO_2 levels to increase in the incubators, thus affecting not only oxygen tension but pH as well.

3.4.2 Electrical System

Much of the equipment in the IVF laboratory is electronically controlled and therefore should have protection against power surges, fluctuations in power quality, and power outages. Any equipment that contains a computer is subject to abrupt, periodic loss of function as a result of changes in electrical current. Although it is now commonplace to have all personal computers on a surge protector or even an uninterruptible power supply (UPS), it is probably even more important to protect laboratory equipment that have microprocessors and perform essential functions.

Two pieces of equipment that may be the most sensitive to unstable power supply are incubators with infrared gas sensors and controlled rate freezers. If there is a power surge or a variation in current during a programmed embryo freeze, the rate of freezing is altered, and this may affect the viability of the embryos. While a surge protector will prevent abrupt changes in power, the controlled rate freezer will not tolerate a power loss, even the short loss of power when the system switches to emergency backup. Thus, controlled rate freezers must be connected to a UPS.

Complete power outages due to a variety of causes are not uncommon and require alternative power supplies. While it is feasible to put critical equipment on their own UPS, most UPS have a limited amount of time for which they can provide power. During a weather emergency, such as a hurricane, tornado, or blizzard, extended power outage can occur and long-term backup power is better achieved by a natural gas or propane-powered generator. Key outlets and lighting, and in some cases the HVAC system, are put on backup generator power. The amount of power required will determine the size of the generator. These generators provide peace of mind but come with their own maintenance issues. Backup generators must be "exercised" on a weekly or monthly basis and their fuel supply maintained. Propane or natural gas tanks are difficult to monitor, so they are commonly changed on a schedule. A better alternative is a natural gas line that should provide constant supply in the event of an emergency. With the advent of vitrification, a further backup for extended power failures is the ability to rapidly vitrify all stages of embryos, thereby reducing the amount of backup energy required.

3.4.3 Alarm System

A system that provides prompt notification when a device fails is critical for IVF laboratories. Like many of the systems in the IVF laboratory, there are several options available. The Sensaphone call-out device is one of the more commonly used systems because of its simplicity. Various models have different capabilities, but a basic unit can monitor up to eight different channels/inputs, including sound in the room. When a variable such as incubator pH goes out of range, after a user-defined delay, the Sensaphone begins calling a programmed list of telephone numbers. It will continue to call the list until someone keys the "cancel" code. While this system is inexpensive and effective, its biggest limitation is that it does not track the actual values. Some newer incubator models periodically record digital readings and alarm history for later display and in combination with the Sensaphone would give an adequate representation of laboratory conditions. Alternatively, there are computerized systems that record all vital data and allow for more thorough quality control.

In addition to electrical and gas outlet placement, careful planning should be used for alarm contact placement. This is becoming less of a problem with new alarm systems that use wireless technology.

3.5 Other Laboratory Features

As technology advances, so do the options available for IVF laboratories. Whereas 10 years ago computers were rarely found in IVF laboratories and some thought they did not belong there, the introduction of electronic medical records

(EMR) and time-lapse imaging has made computer capability in the laboratory a near necessity. Fortunately, wireless technology removes the burden of having to strategically place Ethernet outlets at the time of build-out. The challenge most laboratories now face is providing space for computers. Retractable shelves for laptops are one option, but many times, a hardwired alternative is necessary for computers connected to microscope cameras.

Cameras on microscopes not only are used for documentation of embryos or oocytes for the EMR but also provide an image that can be displayed in the laboratory for teaching purposes or in a waiting room for the patient's partner to view during a retrieval or transfer. In the same manner, it is common to put an intercom system so the embryologist can provide feedback to the physician during procedures.

As each decade since the birth of Louise Brown has brought significant change to laboratory practices in IVF, most recently the addition of time-lapse and blastocyst biopsy, we can expect more changes that will likely dramatically change how space is used in the laboratory. These changes will make some of the topics presented here moot, just as vacuum lines are no longer needed for media preparation and filtration [10].

Introduction of small benchtop incubators during the past 20 years [16] and more recently time-lapse imaging [17, 18] provides a paradigm shift for both space utilization and gas delivery. Detrimental effects of door openings and handling dishes on temperature and pH fluctuations are thus minimized.

3.6 New Construction: Validation Before Patient Care

New laboratory construction is analogous to a new car: the plastics, paints, and adhesives are new and give it the "new car" smell. While some may find this desirable in a car, these odors are VOCs and they pose a serious threat to embryo culture. In order to hit the ground running, it is critical to provide ample time for "burning in," air testing, cleaning, and, finally, quality control testing.

The rate of off-gassing new construction can be increased by "burning in" the facility. Since the compounds of interest are volatile, their rate of dissipation increases with higher temperatures and air exchange rates. Typical burn-in involves increasing the room and equipment temperature by 10–20 °C with high rate of air exchanges for 10 days or more [4, 6]. During this time, all equipment and lighting should be used to assure everything is working properly and enhance the effect of the higher temperatures.

The next step is the first of two tests to determine if your careful design, planning, and oversight of the building process provide low VOC and low particulate "good quality" air that is desirable for an embryology laboratory. Several HVAC tests, including but not limited to VOC testing, HEPA filter verification using particle counts and tracer studies, and pressure verification, will tell you if your collaboration with the engineer and contractor yields the desired results. Specifics for air quality should be in the original contract (see chapter on "Air Quality Management") and if the specs are not met, then you should stop at this point and correct the problem.

Once the facility passes the air quality tests, it is time to proceed with preparing for embryology culture testing. A final cleaning of ceiling, walls, and floors should be completed first. Incubators should have already been running for at least a few weeks or months to burn them in as well.

The final step in the validation prior to clinical use is quality control testing using an animal model. While there are many opinions regarding the utility of different bioassays, the one-cell mouse embryo assay (MEA) has been shown consistently to be the most sensitive, and it appears this sensitivity can be further improved via time-lapse imaging [19] or extending the assay to 144 h [20]. After completing all the aforementioned steps in this carefully planned project, the newly constructed laboratory space should be ready to provide an excellent culture environment.

3.7 Conclusions

This chapter describes many of the critical components of laboratory design and construction, including specifications that at this time are neither standard nor regulated for IVF laboratories. Knowledge of these factors is essential for ART professionals planning a new facility or redesigning an existing one.

Without question, the single most important component of a new facility is the indoor air quality for the IVF laboratory and incubators. Since indoor air quality is influenced not only by the HVAC design and install but the components used for laboratory construction, it is critical that sufficient care is given to the choice of design, materials, and construction. Thorough research and a hands-on approach provide a measure of security for when the laboratory goes "live" – that moment of truth when you witness the fruits of your labor: good fertilization and embryo development and many happy patients.

Review Questions

1. What designs are used to connect the laboratory to the operating room and what are the benefits of each?
2. Alarm systems should be present for which equipment?
3. What can be done to assure a new construction is suitable for patient use?

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Air Quality Management

Johan Guns and Ronny Janssens

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Learning Objectives

- Legislation on ART laboratory air quality
- Qualification, classification, and monitoring of air quality
- Equipment and methods for particle testing
- Methods and frequency of microbiological monitoring
- Prevention, detection, and removal of volatile organic compounds (VOCs)

4

4.1 Legislation

Requirements for air quality aim to prevent (cross) contamination of cells, tissues, or tissue-based products intended for administration to a recipient, as well as exposure of laboratory personnel to communicable disease agents. In the beginning of this century, both the US and European authorities issued regulations to ensure quality and safety of human tissues and cells in general. Concerning assisted reproductive technology (ART), the legislation differs between the USA and Europe and is subject to interpretation.

In 2001, the US Food and Drug Administration (FDA) promulgated rules intended to prevent the spread of communicable diseases by establishments manufacturing human cells, tissues, and cellular- and tissue-based products (HCT/Ps)[1]. As defined by title 21 of the Code of Federal Regulations (CFR) Part 1271, HCT/Ps include semen and other reproductive tissues (21 C.F.R. § 1271.3(d)). The cGTP demands a hygienic environment with strict control of temperature, humidity, and pH and, when appropriate, of air quality. Cleanliness of air implies control of ventilation, filtration, and an aseptic working process guaranteed by cleaning, disinfection, and maintenance of equipment used for this purpose [1]. However, subparts D and E of the cGTP describing the processing of tissues and cells, as well as the requirements for personnel and facilities, are not mandatory for reproductive HCT/Ps [1]. The 2006/86/EC European Commission directives, based on the 2004/23/EG European quality and safety standards for human tissue and cells, do include reproductive tissues and cells in the scope [2]. While aseptic processing is optional in US ART establishments, it is mandatory in Europe. Facilities that process tissues and cells in an open system (air contact) without subsequent microbial inactivation process require EU GMP grade A (ISO 5) air quality for particle and microbial colony counts, as defined in the European Guide to Good Manufacturing Practice (EU GMP) Annex 1 [3]. Additionally, the background environment of the processing zone needs to be appropriate for the cells or tissues concerned, at least EU GMP grade D. Less stringent environmental conditions may be acceptable if the risk of transmitting microorganisms is lower by processing the cells or tissues than by transplanting them in the recipient or if the cooling effect of the grade A laminar airflow is harmful to the tissue or cells. Based on a risk assessment, tissue establishments should specify the air quality of the processing zones and surrounding areas. Several factors such as the particular type of tissue, the processing method being applied, the presence of contaminants, or the final destination of the tissue should be taken into consideration.

Since the implementation of the air quality standards, ART establishments have neglected to perform controlled studies on particulate and microbial air quality. Today, there is still little evidence that particulate filtration avoids communicable disease transmission through reproductive HCT/Ps. Either the risk is really low, or transmissions have been underreported [4]. The concentration of the volatile organic composition (VOC) of the air undoubtedly has an influence on the embryo development and clinical pregnancy [5–10]. However, chemical air filtration, unlike filtration of particulates, is far from standardized [11].

4.2 Qualification, Classification, and Monitoring

4.2.1 Qualification

Qualification of instruments, facilities, utilities, or systems is required to verify their proper function. The qualification activities should consider their whole life cycle from defining the user requirement specifications (URS) through decommissioning. The main stages are defining the URS, installation, operation, and performance qualification. The selection of the test methods used for demonstrating compliance should be based on the user requirements, operating parameters, and selected limits. Requalification can be done after failing, modification, or on a periodic time interval to demonstrate that the instruments, facilities, utilities, or systems are still operating according to the design.

4.2.2 Classification

The classification is part of the qualification process of clean rooms and clean zones and should be clearly differentiated from monitoring operational processes. Classification is a method of assessing the level of air cleanliness against a specification for a clean room or clean zone device (EU GMP, US cGMP) by measuring the airborne particle concentration (Table 4.1). Classification is typically done on a yearly basis by a testing organization in compliance with the ISO14644-1 standard [12]. Classification has to be performed both at rest and in operation. At-rest state does not give any information about the aseptic performance in operation when different sized particles are introduced into the clean room by the personnel and their material and subsequently resuspended from the floor [13]. A worst-case process simulation can be used for operational qualification.

4.2.3 Monitoring

Monitoring of the ART clean room or clean zones can be performed by personnel of the institution, either from the ART, hygienic, or quality departments. Monitoring basically consists

of the enumeration of particles, microorganisms, and VOCs during processing to ensure that the people, process, and the environment remain in control during operation. Europe and the USA apply different recommended microbiological levels (Tables 4.2 and 4.3). Additionally, the measurement of airflow, pressure difference, temperature, and humidity is recommended within ART laboratories.

Before starting, a monitoring program needs to establish the sampling frequency and locations, the number of samples per location, the sample volume, and test methods. This way of working, not yet familiar to ART, derives from pharmaceutical guidelines and other standards concerning clean rooms and associated controlled environments. The ISO 14644-2 [14] and ISO 14698-1 [15] can guide the ART establishments in the setting up of a monitoring program.

Table 4.1 Air cleanliness classification – USA and Europe

Classification			Maximal number of particles/m³
ISO 14644-1 US cGMP USP <1116>	EU GMP at rest	EU GMP in operation	Particle size ≥0.5 µm
ISO 5	A	A	3520
	B	–	3520
ISO 6	–	–	35,200
ISO 7	C	B	352,000
ISO 8	D	C	3520,000

4.3 Practical Implication and Technical Aspects

4.3.1 Particle Testing

4.3.1.1 Equipment

The concentration of airborne particles is measured with a light-scattering discrete-particle counter and includes both nonviable (dust, fibers, skin flakes) and viable (microorganisms) particles. Several light-scattering devices are commercially available. The selection of the device is guided by purpose, frequency, and volume of sampling. The flow rate of a particle counter is crucial since it determines the time needed to collect the minimum sample volume. Particle counters can be designed for continuous or discontinuous measurement. Instruments for continuous measurements are assigned to one specific sample location and mostly used for the monitoring of aseptic environments (EU GMP grade A, ISO5) and their backgrounds. Portable particle counters can be used for both continuous and discontinuous measurements. Their flow rate of 1–3.5 cubic foot per minute (cfm), with 1 cfm corresponding to 28.3 L/min, permits classification and monitoring. Handheld particle counters with a low flow rate, ranging from 0.1 to 1 cfm, are exclusively designed for discontinuous measurements. Handheld particle counters are for time sake less used to monitor cleaner environments such as ISO5 or better. Their main application lies in the localization of the source of airborne particles.

A GMP grade A environment implies continuous monitoring of particle counts [3, 16]. Since probes have to be positioned within a distance of 12 inches (30 cm) of the exposed product in order to give evidence for aseptic processing, as advised for liquid sterile fill [16] and the need for equipment

Table 4.2 Recommended limits for viable airborne particulate – USA and Europe

Classification		Maximal amount of CFU in the environment[a]					
ISO 14644-1 US cGMP USP <1116>	EU GMP	US cGMP		USP <1116>		EU GMP	
In operation	In operation	Active air	Settle plate[b]	Active air[c]	Settle plate[c]	Active air	Settle plate[b]
		CFU/m³	CFU/4 h	%	%	CFU/m³	CFU/4 h
ISO 5	A	1	1	<1	<1	1[d]	1[d]
ISO 6	–	7	3	<3	<3	–	–
ISO 7	B	10	5	<5	<5	10	5
ISO 8	C	100	50	<10	<10	100	50
ISO 9	D	–	–	–	–	200	100

[a]These are average numbers
[b]Settle plate diameter of 90 mm
[c]Recovery rates for aseptic processing environments
[d]It should be noted that for grade A the expected result should be 0 cfu recovered; any recovery of 1 cfu or greater should result in an investigation

Table 4.3 Recommended limits for microbiological contamination on surfaces – USA and Europe

Classification		Maximal amount of CFU on surfaces[a]					
ISO 14644-1 US cGMP USP <1116>	EU GMP	US cGMP		USP <1116>		EU GMP	
In operation	In operation	Contact plate[b]	Glove/ garment	Contact plate[b,c]	Glove/ garment[c]	Contact plate[b]	Glove print
				%	%	CFU/plate	CFU/glove
ISO 5	A	–	–	<1	<1	1[d]	1[d]
ISO 6	–	–	–	<3	<3	–	–
ISO 7	B	–	–	<5	<5	5	5
ISO 8	C	–	–	<10	<10	25	–
ISO 9	D	–	–	–	–	50	–

[a]These are average numbers
[b]Contact plate diameter of 90 mm
[c]Recovery rates for aseptic processing environments
[d]It should be noted that for grade A the expected result should be 0 cfu recovered; any recovery of 1 cfu or greater should result in an investigation

in ART clean rooms is high, portable or handheld particle counters can be an alternative.

4.3.1.2 Sample Volume

The required volume per sample depends on the cleanliness and the functional state of the environment. For monitoring purposes, the minimal sample volume (Vs) is not specified, neither in the ISO14644-2 [14] nor in the European or US GMP guidelines. For classification, however, it is elaborated in the ISO14644-1 [12] guideline as $V_S = \frac{20}{C_{n,m}} \times 1000$. The idea behind this formula is that a sufficient volume of air has to be sampled in order to detect at least 20 particles if the concentration of the largest particle ($C_{n,m}$) were at the class limit.

For example, $\frac{20}{29{,}300} \times 1000 = 0.68L$ is required in an ISO 8 (EU GMP grade D at rest) environment, where the maximal concentration for particles larger than 5 μm is 29,300/m^3 air. The ISO14644-1 guideline also demands to increase the calculated volume to minimal 2 liters within minimally 1 minute sampling time [12].

4.3.1.3 Sampling Locations

For classification purpose, the minimum number of sample locations is defined in the ISO14644-1 guideline annex A [12]. The clean zone must be divided in sections of equal area. Within each section, a sample location should be selected considering it to be representative of the characteristics of that section.

For monitoring purposes, the minimum number of sample locations is not defined in either the ISO14644-2 or the European or US GMP guidelines. Use an appropriate risk assessment tool to understand and evaluate the critical sample locations in function of the air cleanliness required, ART-specific processes, and product locations.

4.3.1.4 Frequency of Particle Testing

Classification is typically done on a yearly basis by a testing organization in compliance with the ISO14644-1 standard [12].

According to pharmaceutical guidelines, a GMP grade A/ISO5 environment implies continuous monitoring of particle counts [3, 16]. Since the risk of transmitting microorganisms is lower by processing reproductive tissues than by transplanting them in the recipient, periodic monitoring of the concentration of airborne particles is acceptable.

4.3.2 Viable Particles

Airborne microorganisms (viable particulate) can be collected, recovered, and grown either after active sampling of a known volume of air permitting quantification or passively using open sedimentation agar plates. Surface imprints on contact agar dishes serve to validate and monitor the disinfection process. Both the EU GMP and US cGMP guidelines specify these methods for the monitoring of viable particulate of air, surface, and personnel [3, 16]. These tests can be performed for different reasons, each requiring a specific approach. Technical details are, however, seldom described in the literature. If so, they apply to the manufacturing of medicines or medical devices and are recommended but not mandatory. The ART institution has to decide which methods to use for microbial monitoring and demonstrate their efficiency by own historical data and risk analysis, often

asked for by inspectors as mentioned by Clontz [17]. In this section, we will discuss how to set up a routine microbial monitoring program for ART clean rooms.

4.3.2.1 Frequency of Microbiological Monitoring

The monitoring frequency depends on the classification: the lower the maximum permitted viable particulate, the higher is the frequency of monitoring. GMP guidelines do not go into detail. The EU GMP asks frequent monitoring, the US cGMP near (e.g., daily, weekly, monthly, quarterly) and long-term oversights [3, 16]. The USP <1116> go more into detail and suggest frequencies for each type of microbial sampling for aseptic processing areas and nonadjacent aseptic areas [18]. It is generally accepted that the processing of tissue or cells in ART implies a significant lower risk of transmitting bacterial or fungal infections to the recipient than embryo transfer. Publications and case reports dealing with infected ART culture dishes are scarce. The added value of routine aseptic process monitoring (in operation) seems therefore questionable. However, this low incidence may be partly due to underreporting. Kastrop routinely performed microbiological examination in all turbid culture dishes for an 8-year period from 1997 onward [19]. He registered infected cultures in 0.68% IVF and ICSI cycles combined, although exclusively occurring in IVF cycles with an incidence of 0.86%. Interestingly, in one third of them, the same microorganism, based on antibiotic resistance, was isolated from the semen. In two-thirds of ART culture infections, the source was elsewhere. Regular environmental monitoring of the process core and background is therefore recommended.

4.3.2.2 Microbiological Sampling Locations

Guidelines for microbial monitoring such as ISO14698-1 and ISO14698-2, US cGMP, EU GMP, or USP do not elaborate the minimum number of locations for microbial monitoring. General information is provided for designing dynamic sample plans based on risk assessment and trending performance. From a practical point of view, it is interesting to combine some sampling locations chosen in the classification plan with locations based on risk assessment in order to design the environmental monitoring plan and use these locations repeatedly for viable as well as nonviable particulate. This gives the opportunity to collect trending data regarding the overall environmental performance of the clean room. However, counts of viable and nonviable airborne particulate are not correlated [20]. For aseptic process monitoring, specific sampling locations must be chosen in function of the process, workflow, and/or, temporarily, incidents.

4.3.2.3 Active Air Sampling of Viable Particles

The USP, EU GMP, and US cGMP consider active air sampling as the standard method for microbiological monitoring of classified environments [3, 16, 18]. Active air sampling, better known as monitoring of airborne viable particles or also bio-aerosol collection, aims to harvest biological particles able to become a colony-forming unit from the air. Active air sampling devices project aspirated air on a culture plate or strip. Preservation of the biological integrity and growth capacity of the microorganisms following impact is critical. Differences in technology and procedures between instruments result in different collection efficiencies [20–26]. Current air samplers are thought to underestimate bacterial concentrations, especially those of single bacterial cells with 0.5–1.0 µm diameter [26]. Technical innovations are expected to improve precision and sensitivity and might change absolute numbers of detected organisms. The choice of an air sampler can be determined by the validation of the instrument, either by the manufacturer or by a third party, in agreement with annex B of the ISO14698-1 that deals with collection efficiency [27]. The cutoff size (d_{50}) determines the collection efficiency of small bio-aerosols and should be as low as possible, considering that one-third of the viable particulate generated by clean room dressed operators is less than 2.1 µm [28].

4.3.2.4 Passive Air Sampling of Viable Particles

In passive air—or sedimentation sampling—open Petri dishes with culture medium are put in the clean room at working height for a specific time period. Following culture, colony-forming units (CFU) are counted. Results are reported in general as number of CFU per 4 hours exposure time [3, 16]. It is a cost-effective and easy to establish monitoring method, unfortunately with some disadvantages. Despite quantitative results, passive air sampling is considered a qualitative method. Since microorganisms deposit on the Petri dish due to airflow patterns and movements of personnel, it does not represent the concentration of airborne particulate. Settling plates have been shown 17 times less efficient than active air sampling, especially in clean environments such as laminar airflow cabinets [29]. Because of the inherent variability of microbiological sampling methods, the USP introduced the incident rate at which environmental samples are found to contain microbial contamination (%) instead of focusing on the number of colonies (◘ Tables 4.2 and 4.3) [18].

The EU GMP and US cGMP advise 4 hours exposure time [3, 16]. In practice, this is not always possible due to dehydration of the culture medium. Dehydration depends on the composition and volume of the culture medium but also on the airflow rate and pattern.

During the validation of our microbiological monitoring program, we studied this phenomenon on 30 mL Tryptic Soy agar plates with a diameter of 90 mm (Heipha Diagnostika reference 030826e) (unpublished data). Petri dishes were weighed before and after exposure in either a horizontal (flow rate of 0.46 m/s) or vertical laminar airflow cabinet (flow rate of 0.40 m/s) for different time periods. As expected, the weight loss relative to the original weight increases with the exposure time and gets to 10.7–11.7% in horizontal flow and 9.3–11.2% in vertical flow after 4 hours exposure. A 13% loss in water content has been shown to be correlated with an 8% loss of viability of test organisms [29], indicating again that each method has its advantages and disadvantages. Passive air sampling is required for aseptic process monitoring in the EU but optional in the USA [3, 16].

4.3.2.5 Monitoring of the Disinfection Program Using Contact Agar Dishes

EU GMP [3] and USP [18, 30] demand surface monitoring of facilities, furniture, equipment, and garment at the end of processing and after sanitation. Surface monitoring can be performed by the contact plate method or by swabbing. In the most commonly used contact plate method, an open Petri dish (diameter of 55 mm) overfilled with culture medium containing biocide-neutralizing agents is pressed against the surface for several seconds, allowing the organisms to adhere to the agar. In case of non-flat surfaces, 24 to 30 cm^2 surface area has to be swabbed [18]. The swabbing method is less standardized and more operator dependent than the contact plate method. Regularly performed surface monitoring shortly after cleaning and disinfection is a cost-effective method to validate the sanitation program, as well as a screening method for outbreaks of spore-forming microorganisms due to the exclusive use of some biocides [31]. The EU GMP [3] encourages the combined use of different types of biocides.

4.3.2.6 Selection of Microbiological Culture Medium

The international standard for biocontamination control in clean rooms and associated environments ISO14698-1 advises the use of a nonselective culture medium, permitting growth of the expected microorganisms and containing additives to overcome the residual effect of biocides and cleaning agents [15]. The in-house flora of an ART environment is predominantly composed of microorganisms indigenous to humans and not different from the one in industrial clean rooms [32]. Therefore, the culture medium has to be appropriately validated for the growth of diverse bacteria and fungi [16]. Both the USP and European Pharmacopoeia (Eur.Ph.) describe several adequate culture media for the sampling and quantification of microorganisms in controlled environments or nonsterile products [18, 33]. The eventual need for a selective culture medium for yeast and molds, such as Sabouraud Dextrose Agar, has been a matter of debate for years [17]. The USP has proposed the Soybean-Casein Digest (SCD) Agar or Tryptic Soy Agar (TSA) as an all-purpose solid medium for the recovery and quantification of most environmental microorganisms in clean rooms, both bacteria and fungi [17, 18]. The use of only one type of culture medium will simplify the monitoring program and minimize the costs. Alternative media as listed in the USP <1116> or Eur.Ph. <2.6> can be used if validated for the purpose intended [18, 33].

Additives inhibiting residual biocides and cleaning agents are an essential component of the culture medium. The choice for a sanitation and disinfection program with a specific cleaning agent and biocide must be well considered. Besides beneficial microbiological control, it can have negative consequences for ART in terms of pregnancy rate [6, 9, 34]. The concentration of residue left on the surfaces after cleaning depends on the type of biocides and the sanitation program. Alcohols, hydrogen peroxide, or peracetic blends deposit fewer residues than quaternary ammonium compounds, biguanides, chlorine dioxide, or hypochlorite-containing agents. On the other hand, alcohols and peracetic blends do generate considerable amounts of VOCs hazardous for embryos, leading to a decreased pregnancy rate [6]. Since low residue concentrations can interfere with microorganism recovery, commercial suppliers offer ready-to-use culture media with a company-specific mixture of one or more neutralizing agents. The diverse composition and concentration of these neutralizing agents makes them incomparable between suppliers. At present, none of the commercial neutralizer is able to inactivate all biocides. Sutton tested 6 neutralizing broths against 13 commercially available biocides, and none of them proved adequate for all tested index organisms (*Trichophyton mentagrophytes, Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus, Clostridium sporogenes, Salmonella choleraesuis,* and *Escherichia coli*) [35]. It is the task of each tissue establishment to demonstrate that the residues generated by their sanitation program do not interfere with microorganism recovery. Different protocols can be used, and among them are the methods of Clontz or Bleisteiner [17, 36]. The former compares the growth of microorganisms recovered from coupons either previously coated with the biocide/cleaning agent or not before inoculation. The recovery phase is performed in the presence or absence of neutralizing agents. In the method described by Bleisteiner, the same number of colony-forming units is inoculated on three culture plates, two of them supplemented with neutralizing additives and one of these also treated with biocide/cleaning agent. Bacterial growth in the plate supplemented with neutralizing additives and treated with biocide/cleaning agent should be at least 50% in the untreated plate supplemented with neutralizing additives.

4.3.2.7 Incubation Conditions

Recovery and incubation conditions have to be compatible with the sampling method in order to permit growth and identification of viable microorganisms and validated as such. Ideal culture conditions differ between microorganisms, 3–5 days at 30–35 °C for bacteria versus 5–7 days at 20–25 °C for fungi and molds [15–17]. Therefore, some centers collect duplicate samples, which double sampling time and reagent costs. Alternatively, one general culture medium such as TSA can be incubated under biphasic conditions [32, 37].

4.3.3 Volatile Organic Compounds

The impact of poor laboratory air on the culture of human gametes and embryos are widely accepted [38–40]. VOCs are natural or synthetic chemicals that can vaporize under normal atmospheric conditions. The compounds that the human nose can smell are generally VOCs. In 1997, Cohen was the first to perform an assessment of chemical air contaminants in an IVF environment and to report a negative correlation between VOC levels and human embryo development [6]. He also showed that unfiltered outside air may

be chemically cleaner than HEPA-filtered laboratory air or air obtained from incubators. The sources of this contamination can be localized outside or in adjacent rooms [5, 6]. Elevated aldehyde concentrations in laboratory air after the use of road sealants to resurface a parking were shown to be correlated with detrimental mouse embryo development [34]. Emissions from motor vehicles and building renovations influence embryo quality and clinical outcome of ART [34]. Other known sources of VOCs are paints, varnishes, petroleum products, pesticides, glues, and cleaning products. But VOCs can also be emitted from materials used within the laboratory. VOCs can accumulate from gasses used for incubators or anesthesia, from plastic Petri dishes, cleaning agents, or alcohols such as methanol and isopropyl alcohol.

4.3.3.1 Prevention

The best policy to reduce the level of VOCs in the laboratory air is prevention, starting with selection of location and in the planning phase of design and selection of building materials for their low VOC-releasing property [41]. Common products used for furniture such as formica, pressed wood, or fiberboard release formaldehyde and should be avoided. Sealants and toxic glues should be avoided. Special attention should be paid to floor coverings, which often are glued and can release VOCs over a long time period. Water-based low odor paint formulated for low VOC content should be used for interior painting [42]. If epoxy paints are required for regulatory reasons, low VOC products are advised.

Ideally, the lab is equipped with an overpressured heating, ventilating, and air conditioning (HVAC) system coupled with air purification systems with active carbon and oxidation using potassium permanganate and/or photooxidation, as well as physical filtration to remove eventual VOCs from outside air. Boone reported effects on fertilization, embryo development, and clinical pregnancy rate, decreasing following construction activity adjacent to the ART laboratory and increasing after moving the ART laboratory to a class 100 clean room with ultra-low penetration air (ULPA) and active charcoal filters as well as ultraviolet light in the air flow [5]. Filter efficiency needs to be monitored on a regular basis since saturated filters gradually release their accumulated VOCs into the filtered air.

Concerning laboratory activity, a strict policy should be adapted to prohibit disinfection alcohol, isopropanol, and ether. Products for cleaning and disinfection of floors and surfaces require special attention. Alcohol-based detergents should be avoided. Nonembryotoxic alternatives are nowadays available based on H_2O_2 or electrolyzed brine. Validation of our sanitation program based on detection of VOC and microbiological counts directed us to the alternating use of an alcohol-free quaternary ammonium compound blended with a biguanide and an alcohol-free amphoteric surfactant for daily floor disinfection. Styrene can be released from polystyrene culture dishes into the air of the incubator [6]. As a preventive measure, disposable culture materials can be removed from their plastic wrapping in a dedicated area, thereby releasing previously accumulated volatile compounds (out-gassing). Material used for incubators releases VOCs. New incubators can contain high levels of VOCs that should be removed before use [43]. Gasses from metal bottles containing compressed gasses can contain up to 60 different VOCs, the most obvious being benzene, refrigeration agents, and isopropyl alcohol [43]. Activated carbon and potassium permanganate in-line filters should be placed on the gas lines to the incubators. Some clinics have a policy to avoid the use of perfume, although, to our knowledge, there are no scientific data to support this, neither could we measure any VOCs being released from staff wearing perfume (unpublished data).

4.3.3.2 Detection of VOCs

There are several methods to detect VOCs in the laboratory air. In order to identify and quantify VOCs, an air sample is required for detailed analysis [6]. Gas chromatography and mass spectrometry of gaseous samples collected in stainless steel sampling canisters [6] permit quantification and identification of organic compounds, but cannot be performed in a routine ART setting due to the complexity of the method and the specific equipment needed. It is offered on a commercial basis in the ART field by, e.g., Alpha Environmental Inc. [44]. The stainless steel vacuum containers are placed in the laboratory for a fixed period of time and sent for analysis. Alternatively, chemical sensor badges [45] can be placed in the laboratory for 24 hours and sent to a company for analysis. In this case, air diffuses through a microporous membrane inside the badge, where vapors are collected on specific absorbents. This technology has been developed for indoor air quality and can analyze more than 100 chemicals. Both the spectrophotometry/mass chromatography and badge methods take snapshots of air quality at a single time point. Multiple measurements are required for monitoring and analyzing variations in VOC composition and concentrations. For regular monitoring, instruments that merely detect and/or measure VOCs without identification can be of help. These instruments, based on photoionization detectors (PIDs) with a broad detection range, are ideal instruments for detecting sources of VOC contaminations in laboratory air. There are no established safe levels in human IVF, but it is a good precaution to minimize the VOC concentration in laboratory air. PIDs in the ppb range are now available, and these can be connected to any equipment monitoring and alarming system.

Formaldehyde in laboratory air can be quantified and continuously measured by colorimetric/photometric portable testing devices with very low detection limits (10 ppb).

4.3.3.3 Removal of VOCs

VOC removal has been shown beneficial in bovine ART. Higher blastocyst rates were obtained in VOC than HEPA-filtered incubators by Higdon [7], while increased pregnancy rates without blastocyst effect were shown in the presence of an intra-incubator air purification unit by Merton [9]. Fertilization rates did not change in either study. VOCs

can be effectively removed by solid carbon and potassium permanganate filters [6]. Commercial systems are available for gas bottle inlets, intra-incubator, as well as whole laboratory air purification since filters can be included either in the central air-handling system or in separate stand-alone units [46–48]. The efficacy of filter-based stand-alone systems in a clean room environment with high air change frequency has to be demonstrated, similarly to the recently developed combined stand-alone systems using charcoal/permanganate filters or active charcoal filtration with HEPA filtration and photocatalytic oxidation, which seem promising in a preliminary report [47, 49]. In existing laboratories without VOC filtering HVAC systems, stand-alone systems can be considered.

Recently, dielectric barrier discharge (DBD) devices with cold plasma technology are developed, which provide air disinfection. They are highly effective in situations where there is a need to reduce airborne pathogens but at the same time reduce particles, aldehydes, and VOCs.

Review Questions

1. What is the difference between clean room classification and monitoring?
2. What is the frequency of environmental monitoring?
3. What technique should be used for environmental monitoring?
4. What can be done to prevent the presence of VOCs in ART laboratories?
5. What techniques are used for VOC removal from laboratory air?

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Organization and Workflow in a Fertility Clinic

Amparo Ruiz

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Learning Objectives

- To understand the unavoidable connection of the workflow with the strategic plan.
- To provide basic concepts to understand that good workflow and organization are key ingredients in customer satisfaction and pleasant working environment.
- To see how the workflow must be based on the main objective of a given company.
- To discuss the organization of the activity, time, and people.

5

Key Points

- The organization of a clinic must be based on the policy of each one, depending on the mission, vision, and values of the company.
- It is necessary to define the strategy of the company to develop all the organization and workflow accordingly.
- The organization and workflow may be a dynamic process, continuously adapting to the situation and conditions of the clinic.

5.1 Introduction

The perfect organization of a clinic, or whatever company, is not uniquely true. It must be based on the policy of each one. Depending on the mission, vision, and values of the company, the organization must be defined.

A strategic plan is essential in each company or clinic, because the entire management and workflow organization must be based on the strategy definition. Any single decision regarding the organization of the activity, staff, and time, must be oriented directly or indirectly, to facilitate the accomplishment of the defined strategy.

Workflow is considered as the set of tasks and the set of people or resources needed for those tasks, which are necessary to accomplish a given goal. The set of tasks will be chronologically organized in processes that the people must follow. An organization's workflow is comprised of the set of processes it needs to accomplish, the set of people or other resources available to perform those processes, and the interactions among them.

Usually, as a fertility clinic grows up, the staff adapts the processes to the increasing tasks, in an informal way. Frequently, people trust in their own memory or the goodwill of their colleagues and, indeed, in slow or standard activity days, it works well, or even, if some tasks are forgotten, the clinic schedule will adjust them.

In a slow activity journey, if the nurse sees a patient sitting without anyone having registered her arrival at the clinic, somehow will find the time to attend her without relevant disturbance in the overall schedule.

However, when the waiting room is full and the doctor is late, it may be felt throughout the organization. When the number of small interruptions outweighs the amount of planned work done in a given hour, that impact is felt in slower progress, lower job satisfaction, and potentially lower quality of care.

It looks that usually all people know what needs to get done. Where organizations differ is in how they do it. The examination of how an organization accomplishes its tasks often concerns the organizations' workflow.

Many workflows are already designed in health organizations, mainly those related to basic and general services, but the more specialized and innovative activities, the less designed workflow. This is especially true in fertility clinics or units, wherein not only new techniques arise continuously but also numerous small clinics grow very quickly. Due to these specific circumstances surrounding assisted reproduction clinics, it is not rare to see workflows that arise organically and evolve in a quite logical and even efficient way. But with the increase of the complexity of the unit, interactions among the different departments, overlapping in schedules, and priorities arise, revealing the weakness of an organizational system, designed without an in-depth analysis based on the company's strategic plan.

One important aspect in the evolution of fertility – and not only fertility – clinics is the necessity of introducing or updating a health-care information technology (health IT) into clinical practice. Of course, it is necessary and has undoubtable benefits. However, the introduction of a new or improved health IT absolutely always causes disruptions in the existing workflows. One of the drawbacks when introducing an IT is the possible resistance to change by many employees. But in the other end is the frequent simplicity of adapting the whole workflow to the way their screens and steps are organized. Organizations that are thoughtful about workflow design are more likely to be successful in adapting to health IT [1].

Nevertheless, in health and other services industries, the patient care and personal knowledge of the patient, and even the feeling in the relationship, is another strong source of information that must be included in the organization and workflow. This valuable information can be lost when poor workflows impede communication and coordination or increase interruptions [2].

5.2 Strategy Definition

5.2.1 Analysis of the Organization Philosophy

Thinking on how to organize all aspects of whatever company, its character marks its style, just as it happens in the way of life of each person. Nevertheless, the resources we have will be key elements in how we apply a style of organization, so let's have in mind that resources are scarce and should therefore be used efficiently [3].

As both material and human resources are limited, you cannot do or cover everything. You will have to choose an option, a path. But whatever option you choose, you will always have to abide by the rules of the game set by the need to use these scarce resources in the best possible way, as efficiently as possible.

In the case of organizations that provide health care, we must bear in mind that these services are not only useful for the individuals who receive them but also for society in general; they have a social nature regardless of whether the provider is public or private. Sometimes, the determining factors of this social service may clash with the purely economic factors [4], making the whole situation more complex. But, apart from this peculiarity, every organization, whether medical or not, be it a Reproduction Unit or otherwise, must have a certain strategy, and this strategy will influence the organization and workflow.

From the business point of view, the strategy describes how a company intends to achieve its objectives with the ultimate aim of creating sustained value over time for its members, clients, and citizens [5].

Thus, any organization that offers a product or service to the market has a particular competitive strategy and is positioned in a certain way before its potential clients and its competitors, and all of this irrespective of whether the organization is aware of it or not.

There are numerous advantages in having a clear strategy, developed through an adequate process for planning, deployment, and control, particularly because it implies that the actions of the organization's different departments act in a coordinated way in order to complete a series of targets set, allowing us to monitor how close we are to achieving these goals. This process is called strategic planning.

5.2.2 Strategic Plan

Although it is not the objective of this chapter to detail the development of a strategic plan, we will mention that there are many diagrams that can guide us in this respect, the most typical and common way to present the aforementioned strategic planning process is through a circular diagram of five stages [6], which are as follows:

1. Initial or strategic thinking phase: Definition of the key concepts of the organization, particularly the mission, vision, and values.
2. Internal and external analysis: Studying the fundamental parts of the company and its environment, aiming to link them to each other. It means looking around us to see where we are, and then, we will analyze internally to see, according to our capabilities and our limitations, where we could be.
3. Strategic formulation: From the above analysis, we will have to decide the strategy to follow and formulate the main objectives. The result of this formulation will be the strategic plan.
4. Implementation of the strategy: Once formulated, the strategy must be deployed. It will seek to identify actions to develop and align the organization's different objectives with the strategy.
5. Evaluation and control: This will consist of defining management indicators and monitoring compliance with the objectives set, so its effectiveness will depend on the management control systems available to the organization. According to the analysis of the data and the deviations produced, feedback can be generated for future reformulations of the strategy and organizational change. For this reason, the strategic planning process is presented as a circular diagram, because this evaluation and control phase provides feedback to the process again.

Any planning process must begin with a reflection on the starting position of each organization. The first thing to do is to know ourselves well and be sure of our starting point. Therefore, the main thing in this phase will be to reflect on each organization's so-called key strategic concepts:

- The values.
- Mission.
- Vision.

Once we are sure of the key factors that influence our strategic choice, we must analyze the environment around us (external analysis) as well as our own internal capabilities (internal analysis) in order to conclude on what we could do, depending on the environment, and what we can do, based on our own means and possibilities.

Thus, we can talk about:

- Differentiation strategies.
- Low-cost strategies.
- Specialization or niche strategies.

Companies that follow a differentiation strategy tend to emphasize aspects such as quality, service, design, technology, brand, innovation, etc., and they invest a lot of resources and efforts into them.

Companies that follow cost leadership strategies seek to offer customers a good product or service at a low price, which does not mean in any way that because the price is low, they give a bad product or service. For this, they emphasize aspects such as large-scale production, efficiency in assembly, and design of the product or service; sharing activities with other companies, the company stops providing part the service, and the individual takes over.

In the case of IVI, we have clearly opted for a differentiation strategy based on achieving maximum patient satisfaction.

5.3 Organization Principles

In addition of the strategic plan, there are some general quality principles that always must guide our organizing actions which, from our strategic point of view, are:

- Patient satisfaction. To achieve optimum patient satisfaction, only two relevant areas have to be considered: to personalize each treatment and to have the best possible clinical outcome.
- Integrated team. To reach an integrated team, it is needed to select the right personnel and to practice the best possible leadership.
- Scientific activity. To do science, a part of economic resources in which we will have to invest, we need to have projects from our professionals, motivating them and helping them by a good coordination.

  In fact, all three can be considered as only one: *patient satisfaction*, since a true teamwork and scientific production are actually improving the patient experience and clinical outcome.

  After a deep study of each organization or company, it is very convenient to find only one main principle that includes our strategic plan and organization principles. It could be cost control, professional prestige, brand recognition, or any other. For IVI it is *patient satisfaction*.

Then, every time we ask ourselves if a given action or organizational change is adequate, we just have to answer if it will increase, directly or indirectly, the patient satisfaction degree, and this applies for any other main principle like the abovementioned: cost control, prestige, etc.

5.4 Organization and Workflow Consequences

Workflow analysis has often been used with the goal of improving efficiency. In response to financial pressure and incentives driving provider organizations, minimizing slack time has become important. Some of the studies discussed below demonstrated the power of analyzing and changing workflow to improve efficiency [7].

Workflow analysis can be used to redesign existing processes. A classic study of this type is Cendan and Good's [8] analysis of the routine tasks of the various members of the operating room (OR) team. They found that there was a wide variability in functions based on clinical and organizational factors. They designed a new workflow based on the analysis and conducted a pilot study. Part of their recommended solution involved defining functions in a more consistent fashion. They were able to improve turnover and improve the mean number of cases handled in a day. A significant factor in their success was their consideration of workflow from both the physician and the nursing perspectives.

It is also possible to improve efficiency by carrying out processes in parallel, rather than improving the efficiency of existing steps [9]. Friedman and colleagues [10] compared the impact of administering anesthesia in the induction room versus in the OR for hernia repair patients. They found that the OR time used by the surgeon decreased without significant impacts on patient satisfaction or outcomes [11]. Harders and colleagues [12] employed a combination of approaches. They used parallel processing and process redesign to improve workflow in a tertiary care center with multiple OR suites. This combination of approaches allowed for a reduction in nonoperative time. Similarly, in a study of trauma teams, Driscoll and Vincent [8] modified task allocation so that standard tasks performed during a trauma code were conducted in parallel rather than sequentially.

5.5 Strategy Implementation: Organization of the Clinic Activity

Taking into account what has been said, it is not difficult to conclude that a management system that takes into account the company as a whole, that has global reach, and that also takes into account nonfinancial elements at the time of decision-making will be much more effective.

The design of good organizational workflow is not simply about improving efficiency. Workflow processes are maps that direct the care team how to accomplish a goal. A good workflow will help accomplish those goals in a timely manner, leading to care that is delivered more consistently, reliably, safely, and in compliance with standards of practice. An excellent workflow process can accommodate variations that inevitably arise in health care through interaction with other workflow processes, as well as environmental factors such as workload, staff schedules, and patient load [7].

Obviously, in a private organization, there will always be the objective of increasing profitability, which is even necessary to guarantee the continuity of the organization and, therefore, of the service it provides and its continuous improvement through the necessary investments. Nevertheless, the ways to achieve it can be very diverse and that is precisely what the management model consists of: the set of decisions and actions carried out by the leadership to implement the strategic directions. Said management will be based on the fundamental strategic principle of each organization, which in our case is differentiation through patient satisfaction.

Management, in being always subjected to strategy, is dynamic; and its development, evolution, and optimization are performed through management control.

According to V. Serra and colleagues [13], management control is the direction technique that consists of:

- Establishing objectives at all responsibility levels in the company.
- Quantifying said objectives through a budget.
- Periodically controlling and evaluating the degree of their fulfillment.
- Taking the appropriate corrective decisions.

We believe this definition to be the most accurate, since it considers the institution of objectives as part of the strategic plan, not as something isolated that is decided "de novo."

According to this reasoning, the first thing to do is to find out what really satisfies patients and not to assume it due to its apparent obviousness: achieving gestation. To know what satisfies patients and what does not – and act accordingly – all that is needed is to ask them. Logically, it will be asked in an organized manner, with questions involving the degree of satisfaction in all the aspects that the patients have experienced, from the waiting time to be assisted by phone, obtain an appointment, or enter the office to the kindness and professionalism of each of the collectives and departments, as well the amount and quality of received information, and many other details.

Furthermore, the collection of data must be simple, and it must be registered in a way so as to permit its later measurement and the production of statistical reports.

It is surprising to discover that some patients – even having achieved pregnancy on their first attempt – can be found to be dissatisfied because they had a feeling of insecurity after receiving seemingly contradictory sets of information, or due to having been taken care of by a physician, they did not expect without previously being informed. It is possible that these patients will not recommend our clinic or may not speak well of it. On the other hand, on numerous occasions, even if gestation is delayed or is never obtained, patients reveal very positive opinions when they perceive the true implication of the entire team in achieving their goal and they feel that they have been very exclusively and personally treated.

Once we have the patients' opinions, we know what satisfies them the most and what they believe needs improvement; but we will have also asked them to score – and we can now measure – the degree of importance that they place on each of the consulted areas. Thus, it may be that a high percentage of patients convey themselves as dissatisfied by the waiting times to enter the office or the operating room, but that they do not consider this such an important aspect; however, a particular percentage of patients may be dissatisfied because they were not allowed to participate in decision-making regarding their treatment and also mark this aspect as one of great importance. Therefore, at the time of deciding improvement actions to achieve the patient's satisfaction goal, those areas that combine the lowest satisfaction index with the greatest importance will be prioritized, postponing other aspects that may be even significantly dissatisfying, but that are not deemed as important by patients, for further assessment.

Surveys can be designed internally or an external, specialized company can provide the service, but it is necessary not only to carry them out: they must be well designed and offer useful statistical reports.

The management designed for the fulfillment of the company's strategic plan and the patient's satisfaction will begin by defining objectives, which must be few and challenging – but achievable – and the management tool to attain those goals is clinic organization, which will differ depending on whether the practice is public, private, corporate, etc., and on the company's philosophy.

Therefore, on a day-to-day basis, the management of the unit, clinic, or company is really about workflow and how it is organized.

The organization of a services industry can be classified in infinite ways, but if we really ask ourselves what we need to organize, the right classification will be:

- Organization of the *activity*: departments and sections.
- Organization of the *employees*: charts and roles.
- Organization of *time*: schedules and timetables, work hours, etc.

Applying this simple classification to a fertility clinic, we will have:

5.5.1 Organization of the Activity

In the majority of clinics, there are many activities and services around the patient:

- Patient attention services such as general information, telephone assistance, front desk, appointment management, billing, etc.
- Office visits: gynecology, psychology, genetics, andrology, endocrinology, etc.
- Nursery attention, blood sample extraction, explanation, drugs administration, etc.
- Laboratories: hormones, serology, semen analysis and preparation, IVF, PGD, etc.
- Surgery: oocyte retrieval procedures, embryo transfers, and testicular biopsies. In some cases, reproductive surgery like laparoscopy and hysteroscopy, etc.
- Administrative services: financial office, price information, private insurance management, and financing.

All these activities must be distributed in departments in a logical way, paying special attention to the interaction between them. This entire distribution must be fit into a larger design that includes other activities that are not provided directly to patients but that are equally important, such as management, research, teaching, or relationships with other external doctors and institutions (Fig. 5.1).

On the other hand, the different activities of the unit not only have to do with the service they provide but must also be grouped according to the types of professionals or collectives that perform them, the treatment to which they belong, or the department that carries them out, as is observed in the example in Fig. 5.2.

Once the activities have been organized in groups according to the different criteria, the personnel performing said activities must be well dimensioned and organized.

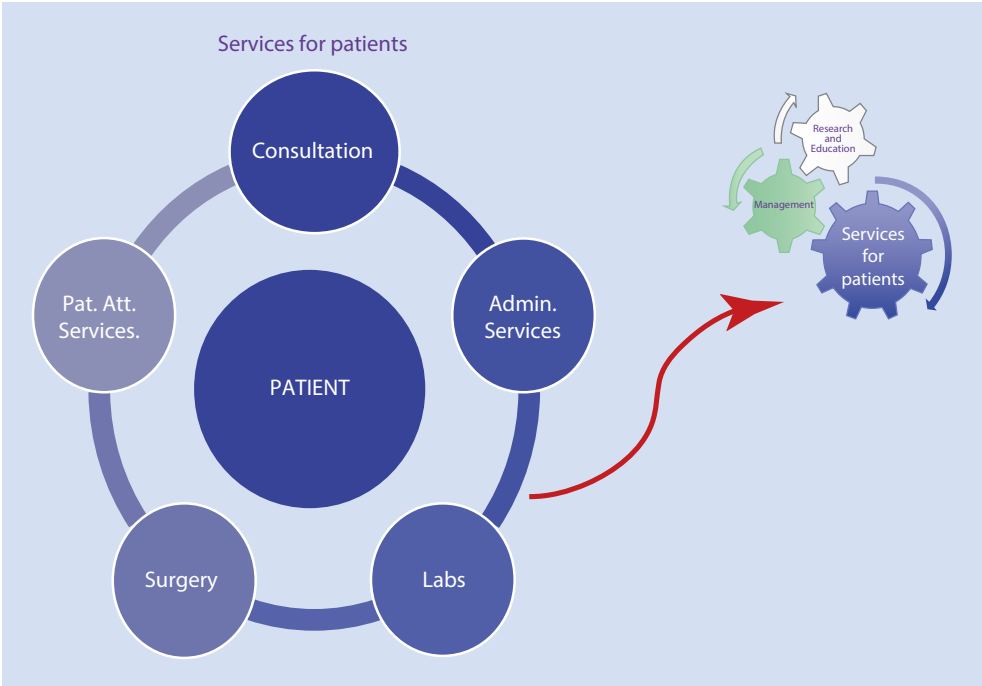

Fig. 5.1 Organization and interaction of activities within different departments

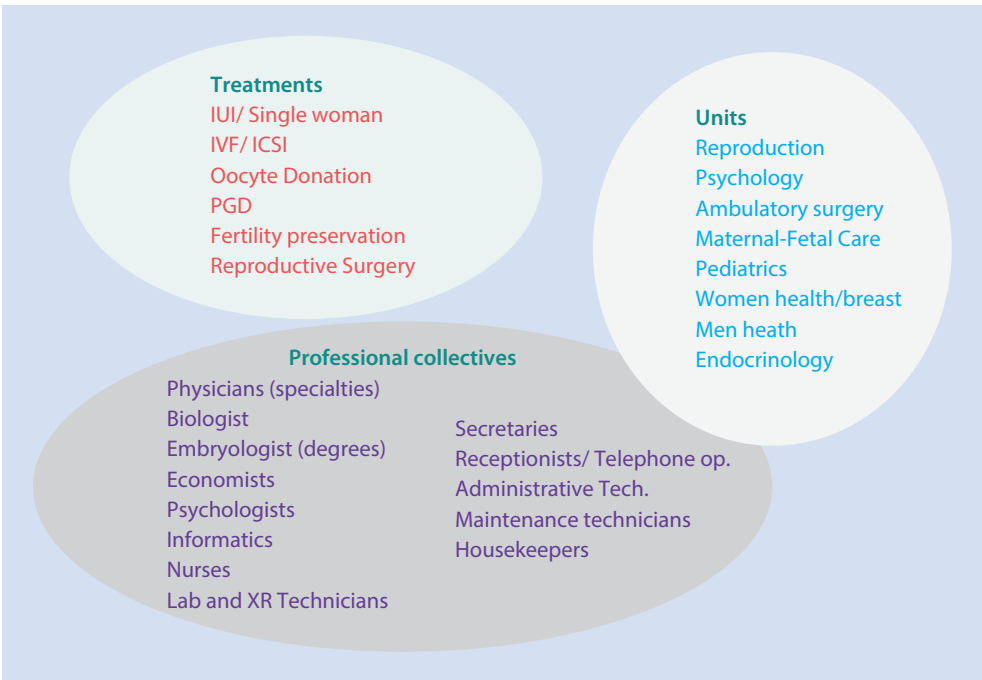

Fig. 5.2 Activities grouped by professional collectives, treatments, and the departments that carry them out

5.5.2 Organization of the Employees

The size of the clinic's staff is a topic of capital importance. If it is scarce, it can result in bad attention to patients and duties, as well as unrest and a bad work environment, which can equally impact productivity and quality. But the staff's dimension is not the topic of this chapter, its organization is.

In any kind of industry with more than one or two departments and more than 15–20 employees, the relations between people within the organization are arranged in an organizational chart. This is a valuable way to enable one to visualize the complete organization, by means of the picture its presents.

By definition, an organizational chart (OC) is a diagram showing graphically the relation of one official to another, or others, of a company.

Frequently, companies use to design an OC adjusted to their actual size and the employees they have at that particular moment, but then, it becomes obsolete when the size, complexity, and number of employees increase, adapting the OC on the fly, resulting in inconsistent perceptions of

reporting lines of the operational teams, as well as confusion in the roles and departments' definition.

In our experience, as a group of numerous different size clinics, it is more convenient to deploy a complete organizational chart – even for small clinics – that could be easily adapted to the growth and increasing complexity in the future. In new or small IVI clinics, one person assumes several roles in the chart and – as the volume and complexity increase – there is already a plan on how to allocate new professionals.

Organizational charts must be designed with vision in the medium or long term, because at the beginning, or in the smallest clinics of a group, one person may assume several roles, but when it grows up, all specific positions are already defined, as well as their hierarchical dependencies. This facilitates maintaining a continuous workflow along the time.

Regarding the hierarchical pyramid, prioritizing the internal communication before than the "command and control," we prefer it to be as flat as possible since this facilitates internal communication and teamwork. In our experience, what works best is a three-hierarchical-level pyramid:

1. Direction level: clinic and medical director, administrative responsible, clinic supervisor, etc.
2. Mid-level management: one manager for each functional unit or department (e.g., IVF Lab, Andrology Lab, Egg Donation, etc.) and one coordinator for each professional collective, even if they belong to different departments (e.g., nurses, technicians, front desk – patient services, international department, etc.).
3. Rest of the employees: every single person in the clinic has a representative in the mid-level management.

This structure allows effective communication and rapid diffusion of novelties or changes, since it facilitates internal communications and teamwork.

5.5.3 Organization of Time

Most clinics organize patient flow according to their doctors' individual plans and very few establish their doctors' and departments' schedules based on patient preferences.

Visiting reproduction units and clinics all over the world, in general, it is observed that in most of them, time organization begins by taking into account the different activities, needs, and preferences of the doctors and other collaborators, to then try to fit the patients around that. For example, there are doctors who can have parallel activities that make them only available at certain hours, or the laboratory requires that all blood samples for the determinations must be in by a particular time of the day, or the follicular development ultrasounds are only performed by one person and must all be grouped into a few-hour shift, or the entire team directly wants to have free afternoons, etc., and taking into consideration all these variables, patient flow, office appointments, and operating room schedules are organized.

In an organization based, for example, on cost control, the mentioned system may make sense, although other factors would have to be considered as well. Certainly, however, if our strategy is differentiation based on patient satisfaction, this approach is frontally opposed to what we need.

Following our plan to manage through clinic organization and placing our focus on time organization, in addition to the regulations and habits of each country, it is important to analyze the preferences and needs of the patients to optimize each activity's efficiency. In this line, we define the number of hours per day for patient attention, the patient distribution, or density along the day, whether it will be convenient to see patients until the late hours because that is what they request in some places (such as Spain) to avoid giving explanations at work, or whether appointments for first visits should be offered on Saturdays (e.g., in Japan).

All the working hours of our laboratories, of our personnel, the establishment of the different shifts, etc., will be based upon this information in order to facilitate a complete service to the patient, at the hour that the patient prefers, and with enough staff to be seen without waiting lists or delays at the clinic.

We also measure precisely the mean duration of each type of procedure to assign it the appropriate time in the schedule. These calculations allow us to design the appointments, since it is necessary to minimize the waiting time for the patients and the bad work distribution for the professionals: the objective is that they do not have to wait for us and we do not have to wait for them.

Finally, although we have focused on the term "patient" because we are devoted to a medical activity, private and corporate IVF units must be viewed as service companies. Therefore, in terms of management, when we speak of patient satisfaction, we should really see it in a broad sense of client – and not only patient – satisfaction, which includes both the external client (the patient) and the internal one (the staff).

The organization and the concepts described are applicable, therefore, to the satisfaction of all the people who integrate the team and to the relationships with providers, affine institutions, and societies in which we play a part, in the sense that satisfying relationships – in the long run – help to achieve goals and to be coherent with our strategic plan.

Last but not least, no matter how deeply rooted an IVF unit's organization is, it must be a totally dynamic thing, always remembering that what we do not ask or we do not measure, we do not know. And what we do not know, we cannot improve.

Review Questions

1. On what should the organization of a clinic be based?
2. What should be defined before developing all the organization and workflow?

3. Five steps of a circular diagram for the development of a strategic plan.

4. What are the three key strategic concepts to start any planning process?

5. What is management control?

6. What are the three main areas to organize in a services industry?

7. Is the organization and workflow a static document or a dynamic process? Why?

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Risk and Safety in the IVF Clinic

Julius Hreinsson and Kia Borg

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Learning Objectives

This chapter aims at informing the reader of the following:

- Conceptual information on risk management from a quality control perspective
- Practical information on how to perform a risk assessment
- How to minimize risk and maximize safety in the IVF clinic

6.1 Risk Management: Background

6.1.1 What Is Risk?

All definitions of risk involve concepts of uncertainty and loss or injury. Here, we define risk as a possibility of an activity causing injury or damage to someone's interests. In the context of ART, "someone" is any of the stakeholders in the activities of an ART clinic.

6.1.2 Risk and Conflicts Between Interests

There are inherent conflicts of interest between stakeholders, so that the pursuit of one interest creates risk to another interest, i.e., it increases the probability of injury to another party. For example, the patients (the prospective parents) may desire to have twins, in the belief that they will get "two for the cost of one." However, this is a disadvantage to the offspring, since there is a significant health risk associated with being a twin sibling. The different attitudes to twin pregnancies in Europe [1] and the USA [2] illustrate different ways of solving this conflict and balancing the risks. The community may make a different risk–benefit assessment than scientists when new reproductive technology is about to be introduced. This was demonstrated in Italy, where the legislature severely restricted the practice of reproductive medicine after public reactions to claims of human cloning and egg donation to very old recipients [3]. Finally, there is the conflict between owners and employees, where the two are making demands on the same resource, i.e., a conflict over how the economical surplus from the clinic's operations should be shared between the investors and the employees. If either party receives too little in return for their input, they may decide to withdraw their support for the organization. Risk management is about balancing risks to different stakeholders, i.e., patients, offspring, community, the profession, owners, and staff.

6.1.3 Types of Risk

The severity of the injury may be anything from a minor inconvenience to the loss of life or the closure of a clinic or even the prohibition of the practice of reproductive medicine. The damage can be financial or nonfinancial (human), and damage to a human interest can lead to financial damage and vice versa. The risks that patients face are both financial and human, i.e., they may waste hard-earned money on inappropriate diagnostic or therapeutic measures, and they may suffer physical injury from the treatment in the form of ovarian hyperstimulation syndrome, infections from bowel perforation during oocyte retrieval, etc. The medical adverse events may also have financial consequences for the patient, and for the treating clinic, as they may be sued for damages. As mentioned above, there may also be consequences for the whole ART industry. Conversely, if the clinic is under financial strain, it may choose to increase risk taking with regard to quality of care, and this may in turn cause injury to patients.

6.1.4 Risk and Quality Control from the ISO 9001 Perspective

Risk management has over the years become an ever more central part of continuous improvement of patient safety and is of importance for the clinics' future progress and development. Risk management is now an integral part of the ISO 9001:2015 certification standard. One of the key purposes of a quality management system is to act as a preventive tool. Annual risk analysis of operations and similar analysis of new and planned activities are advised. This could include organizational changes, application of new methods and techniques, suggestions for improvement or experienced risk for nonconformities, as well as adverse events in other operations. Initiation of risk analysis is typically initiated by management or quality control officers.

6.2 Quantifying and Analyzing Risk

6.2.1 Assessing Risk

The risk perspective helps us see opportunities.

Assessing risks is an essential part of the patient safety work. We can try to avoid risks, reduce risks, or sometimes even accept risks that we cannot do anything about. Risk perspective also helps us to see opportunities for improvement.

We can identify our risks in annual risk assessments and, if necessary, in several specific risk analyses. The identification may be of a general nature such as "worst-case scenario" for the business in general or possible specific risks to a particular process.

Prioritizing is important as it helps us to allocate resources to where they are of most utility. This requires us to quantify the different risks (risk scoring) that can be done by using a "Risk matrix" (Table 6.1).

An example of a risk with very low probability but high severity is death after IVF treatment. Although this is a very rare event, probably occurring at a frequency of <1/100,000 [4], it is a very severe event, obviously not only to the patient and relatives but also to the reputation of the clinic and to the industry.

Table 6.1 Risk matrix

Risk matrix					
		Severity			
		Very severe (4)	Serious (3)	Significant (2)	Minor (1)
Probability	Highly probable (4)	16	12	8	4
	Probable (3)	12	9	6	4
	Less probable (2)	8	6	4	2
	Minor (1)	4	3	2	1

Examples of probability and severity

Probability:

Highly probable (4)	May occur every week
Probable (3)	May occur a few times a month
Less probable (2)	May occur a few times a year
Minor (1)	Occurs very rarely

Severity

Very severe (4)	Death after IVF treatment, misidentification of gametes, transmission of infections
Serious (3)	Inadvertent embryo demise, thawing an embryo on the wrong day, bleeding or critical loss of blood pressure during a procedure
Significant (2)	Wrong stimulation protocol, missed diagnosis of a twin or triplet pregnancy
Minor (1)	Discomfort/concern for the patient, prolonged waiting times, patient not treated with respect

Errors in appointments booking represent a common event, but not a severe risk. In a single instance, they do not have a major impact on the clinic, but if they occur too often, they will damage the reputation of the clinic, and patients may make demands for compensation.

When assessing risk, we cannot limit the analysis to one event going wrong. Instead, we must consider how a minor error in one step can increase the likelihood, or compound the effect of an error later in the process.

Of course, all very severe risks must be addressed immediately as they can threaten the continued existence of the clinic or the life of a patient. However, also less significant events that are likely to occur often should be addressed, as they will drain the resources of the clinic. The risk scoring process only indicates the priorities but does not suggest how to address the risk issue.

6.2.2 Managing Risk

In principle, there are three ways of managing risk: elimination, reduction, and transfer. In real-life situations, it is not possible to eliminate the risk of an activity, except by desisting from that activity. That in itself creates other risks, and it can sometimes be useful to evaluate the options using the matrix below:

What will happen if we take this action	What will happen if we do not take this action
What will not happen if we take this action	What will not happen if we do not take this action

For example, a clinic may choose to not do blastocyst culture because of possible negative developmental effects of

extended culture. One adverse consequence would be that the chances of pregnancy in a fresh cycle decreases, so that the patients may have to undergo more treatments and subject themselves to more treatment risks to achieve pregnancy. Another risk is that the clinic may forgo an opportunity to improve its standing in league tables and then suffer a reduced demand for its services.

Risk reduction requires a thorough understanding of the process and a thorough risk analysis. This involves validation, process mapping, and monitoring of performance indicators for the process in question and the application of techniques like FMEA (failure mode and effect analysis) and root cause analysis. The results of these analyses have to be fed back into the process design or review, and they have to be repeated as experience accumulates.

The concept of risk transfer or risk sharing is meaningful only in the context of financial risk. A very common example of risk transfer is insurance, whereby the insurer bears the brunt of the financial burden of an adverse event. An example of risk sharing is the reimbursement policy that many clinics have for situations where a treatment cycle has to be cancelled or is unsuccessful.

6.2.3 Analyzing Factors That Influence Risk

There are a number of tools that can be used to identify factors that increase or decrease risk. In IVF, the actual probabilities of various adverse events are not well known, and therefore, a qualitative approach is required. One such approach is force-field analysis, whereby factors or forces that increase or reduce risk are identified and ranked. The example shown in Fig. 6.1 is by no means comprehensive.

6.2.4 Monitoring

When the procedure is being performed, the operator should certify that all the steps have been carried out according to the SOP. Witnessing should be certified, electronically or by handwritten signatures. The certification should be audited regularly, and all nonconformities, adverse events, and near misses should be recorded in the incident log, both on a process level and an individual operator level. All incidents must be reviewed and analyzed regularly and system improvements initiated. Training and education may also need to be improved.

6.2.5 Implementation

1. Identify risks: Choose the process you want to analyze and describe the subprocesses schematically in a risk analysis spreadsheet (Table 6.2). Identify risks, for each step of the process, by asking yourself the following questions: "What could have gone wrong in this step?"—"What would the consequences be for the patient?"
2. Estimate the magnitude of risk: Risk size is an aggregate of severity and likelihood of occurrence. Multiply the value of the severity with the value of the probability (see Risk matrix, Table 6.1). At risk score ≥8, an action plan should be developed to reduce the risk. After completion, a follow-up and a new risk estimation are done. Action plans can also be initiated for scores under 8, if needed.
3. Identify the underlying causes: Each risk can have one or more causes. At each risk ask "Why?" and answer "Because...." repeatedly. When the cause (one or more) is identified, the answer to the question "If the identified

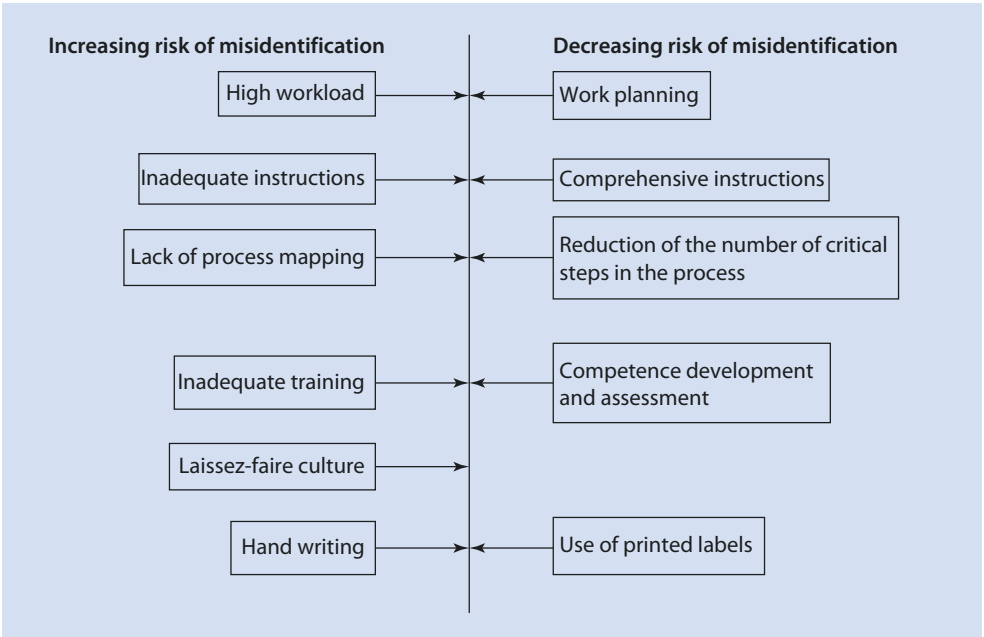

Fig. 6.1 Force-field analysis. One approach to identifying factors that increase or decrease risk is force-field analysis, whereby factors or forces that increase or reduce risk are identified and ranked. The example shown is by no means comprehensive

Table 6.2 Risk analysis spreadsheet

Process:_____

Sub processes	Risk * What happened or could happen? * Where and in what situations it is most likely to happen?	Scoring			Continue the analysis? **Yes or No** If yes, please complete an action list
		Severity	Probability	Risk score	
Description	Description of risk				

Example of a spreadsheet used to identify and evaluate risk in various processes

causes are removed or restricted, will it prevent that the risk may occur again?" should be "Yes."

4. Create action plans and follow-up methods: Give suggestions for actions for each underlying cause to be eliminated, limited, and/or monitored. The suggested action plans should be concrete, realistic, and possible to implement within a reasonable timeframe. Decide a method of follow-up to ensure that the actions have had the intended effect.
5. Prepare a final report: Write a report summarizing the main risks associated with the underlying causes and suggested actions. The report should be objective and based on patient safety.

6.3 Risk Management in the Laboratory

To address a risk, one can follow a four-step method: process analysis with identification of critical steps, identification of how errors can occur in the critical steps and what their consequences could be, possible causes of these errors and their prevention, and determination of how the critical steps should be monitored. This is of course an iterative process. The risk analysis will often lead to the whole process being redesigned, and this then requires a new risk analysis. Also, once the process has been in operation for a while, performance data will be used for a risk analysis to redesign the process.

It is extremely important to focus on the process and not the people. Certainly, individual performance must be monitored and deficiencies addressed, but identifying system weaknesses and opportunities for improvement is the only way to improve by design rather than by chance. It is also important to understand that in a reasonably well-run clinic, improvements happen at a modest rate. The long-term rates of improvement in national live birth rates in Sweden and the UK are in the order of 0.3–0.4% per year. If your clinic improves by 0.6% per year, you are outpacing your average competitors very fast.

Example: Misidentification

Misidentification is one of the most dreaded adverse events. It has a reasonably high probability of occurring and the consequences to the patients, the offspring, the embryologist, the owners, and the profession are dire [5]. Any nonconformity in this area, even if it is discovered before any damage has occurred, must be recorded and treated as a serious incident, analyzed, and acted on.

When designing a process, we start with process mapping. The process map is then used to write instructions for performing the steps of the process, standard operating procedures (SOPs). An SOP should contain background information with scientific references, requirements for performing the procedure (operator competence, materials, equipment, conditions), detailed instructions, requirements for data input and output/reporting, interpretation of results, standards and reference ranges, risks associated with the process, and instructions for nonconformance events. Figure 6.2 is an example of a simple process map for ovum pick-up (OPU) (each step requires elaboration) to assist in risk management of the process.

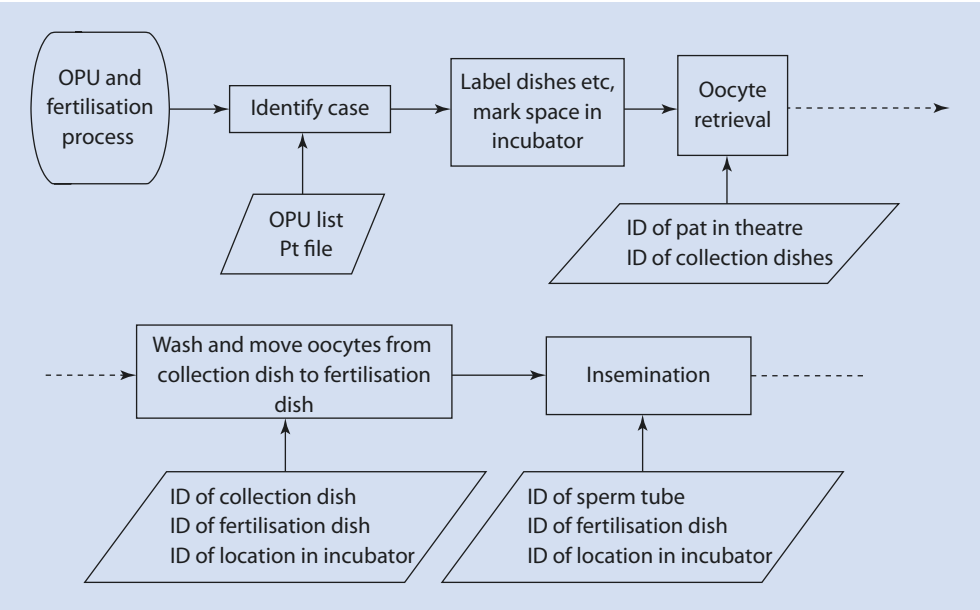

Fig. 6.2 Process map. An example of a simple process map for OPU (each step requires elaboration) to assist in risk management of the process. OPU, ovum pick-up

6.3.1 Where Can It Go Wrong and How Can It Go Wrong?

It is obvious that there is a risk of misidentification in each step. We can misread the OPU list and get information from the wrong patient files. If data for the wrong patients are entered, all ID steps down the line will be incorrect. At another step, the dishes can be incorrectly labeled, because we are working with the wrong information or because we misread the information or because we accidentally labeled the dishes wrongly or in a way that allows misinterpretation. We can put the dishes in the wrong location in the incubator, we can get the wrong set of dishes out, and we can inseminate with the wrong sperm. In short, every time we read something, write something, or move something, we can make an error. If the errors are discovered at an early stage, injury may be limited, although trust is always damaged in these events.

6.3.2 What Is the Cause of the Error?

In this example, the root causes are failure or mistake in reading or writing and misplacing things. A system-level action to reduce errors should therefore focus on these issues. Thus, we should reduce the number of instances where reading and writing is performed, e.g., by transferring information electronically from a patient file onto labels for dishes. By using clear labels and by working with material from only one patient at a time in a workstation, we reduce the risk of misreading and misplacing. When it is necessary to work on material from several patients in parallel, like in sperm preparation, separate workstations for each patient can be used. This increases time cost and equipment cost, thus hurting other interests, so an informed decision has to be made about what level of risk is acceptable and at what cost. Today, RFID (Radio Frequency Identification) and barcode systems for IVF laboratories are available to reduce the risk of misidentification, misreading, and misplacing. Double witnessing may also reduce the risk of misreading and misplacing and is mandatory in IVF in the UK. It is also required in the many processes in the pharmaceutical industry.

6.3.3 Risk Tolerance

Since we cannot eliminate risk but only reduce it, we have to decide how far we want to reduce the risk, i.e., what risk level we can tolerate. There is a cost associated with risk reduction, and some kind of cost–benefit analysis is required for informed decision-making. It is important to estimate the cost of failure, not just catastrophic failure where the cost is infinite but also the cost of minor failures, in terms of time, money, and mental energy. This process is not an exact science, but it is often possible to determine whether a particular action increases or decreases a particular risk or cost. As pointed out above, any action or lack of action involves risk to more than one stakeholder, so the risks to the different stakeholders must be arbitrated, and acceptable risk levels defined, i.e., optimized.

6.4 Critical Decisions Made in the IVF Laboratory

Examples of two decisions, critical to the outcome of the treatment, which are made in the IVF laboratory, are (1) choice of fertilization method and (2) selection of embryos for transfer and cryopreservation. The consideration in the first case is the risk of fertilization failure versus the less known risks of using a more invasive technique as well as the increased costs of ICSI. In the second case, the embryologist must balance the risk of treatment failure against the risks

associated with multiple pregnancy. Each clinic has to develop their own policies based on own data and published information, as well as the risk tolerance in their setting.

6.4.1 Fertilization Method

The use of ICSI varies tremendously between countries. According to the EIM results [6], the frequency of ICSI in European countries in 2011 varies between 36% and 96% with an average of 68%. Certainly, the choice between IVF and ICSI is not always easy for the IVF laboratory, and in many cases, a drift toward an increasing rate of ICSI can be seen. It is, however, well established that ICSI improves outcomes only in cases of low sperm counts or previous fertilization failure in IVF and is not indicated in cases of female pathologies such as poor ovarian response or in cases of previous implantation failure [7–9]. ICSI is more costly than IVF, so this is an economical issue for the IVF laboratory as well.

Each IVF laboratory must develop criteria for selection of fertilization method based on the clinic's patient population, culture methods, and, of course, the quality of the sperm sample. Common indications for ICSI include reduced sperm sample parameters (concentration, motility, etc.) in the original sample and/or low yield after preparation (<1 million motile sperm). Adhering to set parameters increases reproducibility and allows improvements through experience and data analysis since the lack of selection parameters hinders learning by experience—nonexistent parameters cannot be improved. It is important to realize two aspects of this problem:

- Fertilization failure after ICSI occurs at a rate of about 2%, and in the authors' experience, it need not be much higher in IVF, about 3% (unpublished observations).
- Patients with fertilization failure after IVF in spite of a normal sperm sample have a reduced chance of success with ICSI compared to ICSI for male factor patients [10].

The problem is one of informing the patient of the chances of success and limitations of the methods available, based on robust scientific evidence. The IVF laboratory must continuously monitor its key performance indicators, such as fertilization rate after IVF or ICSI, and benchmark them against leading clinics. Over time, this will assist clinics to improve and fine-tune selection criteria. These aspects are discussed in Chapter 70 in this volume.

6.4.2 Selection of Embryos for Transfer and Cryopreservation

This decision involves determining firstly which embryos can be transferred and frozen and, secondly, how many should be transferred. The criteria used for assessing the developmental capacity of embryos include morphology, developmental rate, and metabolic markers, and these criteria can be assessed at a single stage or at multiple stages. As a rule, the earlier the assessment is made, the poorer the predictive power, but by waiting until later stages, the exposure to the in vitro environment is prolonged.

The embryologist thus has to balance the risk of making a poor assessment against the risk of damage to the embryos due to the suboptimal culture environment. Also, the more thorough the assessment, the greater the exposure of the embryos to the environment outside the incubator. If the embryologist is skilled in assessing embryo developmental capacity, the number of embryos transferred can be decreased, and the risks associated with multiple pregnancy can be accordingly reduced.

6.5 Safety and Health in the IVF Lab

As in any laboratory, there are general risk-reducing measures that must be in place to ensure the safety of IVF laboratory staff. These include fire safety planning, instructions for handling any substances, materials, etc., that may cause injury, reducing the risk of slipping and falling, minimizing the risk of falling equipment, etc., and ensuring manual handling is done in an ergonomically sound way. Guidelines for such measures are published by national or supranational bodies, for example, the European Agency for Occupational Safety and Health. In addition, there are health and safety risks that are specific to particular areas of work, and examples of such risks are given below.

6.5.1 Liquid Nitrogen

In the USA, deaths from liquid nitrogen (LN_2) asphyxiation in a variety of industries occur every year, so this remains one of the most severe health risks to IVF laboratory staff [11]. A review of the health risks associated with LN_2 handling in ART laboratories has been published by Tomlinson [12], and a few of the issues are highlighted below.

Because of the huge temperature differences between LN_2 and the laboratory environment, materials such as plastic containers will contract/expand depending on whether they are being placed in or taken out of LN_2. This stress may cause cracking or, in the case of sealed containers, explosive destruction of the container. Use of appropriate materials and containers intended for use with LN_2 is essential to avoid injury and/or loss of specimens. Safety glasses or face shield must be used at all times when handling LN_2 since splattering may occur in addition to the abovementioned risks. Protective clothing and shoes are warranted, especially when handling LN_2 in pressurized containers such as some variants of controlled-rate freezers.

Liquid nitrogen has no smell, and when it evaporates, it has the same density as air since the atmosphere we breathe is to approximately 78% composed of LN_2. When large volumes of LN_2 evaporate, the oxygen (O_2) concentration in the room may drop from the ambient concentration of approximately 21%. One liter of LN_2 will expand in a ratio of 1:694 to the gas phase, so this effect may be quite dramatic. Only a few percent drop in the oxygen concentration may have an effect

on a person in the room, a drop below 10% O_2 will cause unconsciousness, and an O_2 concentration of less than 6% is lethal. Most alarms for low O_2 concentration are set to go off at a level below 18% O_2.

All facilities where large amounts of LN_2 are stored must be equipped with adequate ventilation and an alarm system for a drop in atmospheric oxygen. Detailed routines for each laboratory will depend on the amount of LN_2 stored as well as local ventilation systems. Laboratory directors should remember, however, that even the best ventilation systems may fail occasionally and the most robust vacuum isolated storage tanks may develop an isolation breach or leakage. Therefore, alarm systems are mandatory unless very small volumes of LN_2 are handled. The risk of asphyxiation from displacement of oxygen is present also in any space where CO_2 or N_2 gas bottles are stored, and the same precautions are mandated there.

6.5.2 Ergonomic Aspects of Laboratory Work

The embryologist has a repetitive job where several hours each day are spent at the microscope or performing repeated movements such as pipetting or micromanipulations. The IVF laboratory invests a large amount of training in each employee, and an optimal work environment is necessary to ensure a healthy and productive working life. This is cost-effective in the long run since ill health and financial liabilities can be avoided. Some important issues to consider are the following:

Adjustable ocular tubes for microscopes (ergotubes) are important to ensure an optimal posture while working with analysis and manipulations. This will reduce the risk of back problems associated with long sessions at the microscope. Readers who are not convinced may test sitting in the driver's seat of a car, which has been adjusted for a person 10–20 cm shorter or taller than themselves and drive for 3 h without adjusting the seat or steering wheel. This is equivalent to an ICSI session at a busy IVF lab or a difficult case of TESA-ICSI.

For the same reasons as above, laminar flow hoods with adjustable stands are preferable in the IVF lab. Producers of modern laminar flow hoods should be able to deliver these with height-adjustable stands. Other ergonomic support items include mechanical pipettes and multi-dispensers to minimize repetitive pipetting and semi-stationary arm supports, which may be used, especially if problems have already occurred. Similar considerations are relevant when choosing the height of incubator supporting tables, etc.

6.5.3 Infected Specimens

The risk of infections from infected specimens is not greater in the IVF lab than in other medical laboratories. Patients are screened for hepatitis A, B, HIV, etc., but nevertheless, the use of protective gloves is mandatory when working with samples such as sperm and follicular fluid. Hospital and/or cleanroom clothing should be worn at all times. The use of protective gloves, when performing micromanipulations and handling of oocytes and embryos, may be considered a risk in itself and is not mandatory when working with material from screened patients. Samples from patients with known contagious diseases, on the other hand, must be processed with special caution, using a face mask, single-use protective clothing, and gloves. This type of work must be conducted in designated biohazard facilities, which can be easily and thoroughly cleaned.

Care must be taken when handling sharp instruments such as needles and micromanipulation pipettes as well as breakable items such as glass. Routines should be developed for handling any situation that involves injury to patient or personnel. The routines should include blood testing to check for development of infection and reporting to authorities according to local regulations. It is strongly recommended that the infection status of staff is checked at the start of employment and that vaccination is offered as appropriate.

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Review Questions

1. How can a risk evaluation be performed using a risk matrix where values of probability and severity are taken into account?
2. Why is an O_2 alarm mandatory in storage facilities for liquid nitrogen? Explain.
3. Why is it important to focus on systematic causes of errors in any given process instead of conferring blame to individuals?
4. What are the different kinds of burden conferred on a clinic by infrequent and serious errors vs. frequent and minor errors, respectively? Explain.

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Control of Variables

Cecilia Sjoblom and Georgios Liperis

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Learning Objectives

- To provide an update on core variables within the embryology laboratory
- To discuss and determine ways of improving monitoring to allow for detection of fluctuations
- To provide knowledge and tools to enable action to be taken against the root cause of fluctuations, allowing for quality control process development
- To apply these approaches to implement efficient control of variables

7.1 Introduction

7

Success of assisted reproductive technology (ART) is influenced by a number of different factors. The aetiology and age of the patient seeking treatment, ovarian reserve, response to ovarian hyperstimulation and receptivity of the uterus affect the outcome. The embryology laboratory itself has a leading role, with treatment results being highly dependent on the quality of the laboratory environment, culture protocols and media used, the equipment, disposables and supplies as well as the individual skills and performance of the embryologists [1, 2]. All of these factors can be considered as variables, and while each patient is different to the next, we must strive towards keeping these factors constant in order to increase the success of ART.

The female reproductive tract provides an optimal environment for the gametes and developing embryo. From the point of ovulation, to fertilisation and then as the embryo transverses the oviduct towards the uterus, it is exposed to an environment evolved to support and promote its growth at every stage. The physical aspects of this environment are perfectly controlled with a precise temperature, pH and absence of light. This stands in stark contrast to the in vitro environment of the embryology laboratory, where the main purpose is to maintain the quality of gametes and embryos by mimicking the highly stable environment of the female reproductive tract [3, 4]. The human pre-implantation embryo does not possess mechanisms to respond to changes and stress as adult cells do. Most notably, embryos cannot control changes in physical parameters such as temperature, pH and osmolarity. As a consequence, when embryos are exposed to minute changes in physical and chemical conditions such as media composition and pH, temperature and osmolarity, they are unable to develop optimally [5–7].

7.2 Variables

7.2.1 Temperature

Temperature is a significant variable that affects the outcome of an ART cycle. The concept of temperature control goes beyond the culture dish, encompassing laboratory room temperature and temperature of refrigerators and freezers. During culture, temperature is a key determinant of gamete viability and embryonic growth [8]. Maintaining a constant temperature in vitro close to the human body temperature of 37.0 ± 0.2 °C is crucial during all in vitro manipulation and culture [9, 10]. Modern IVF laboratories control temperature by using incubators with precise temperature and heated stages to allow for temperature control during manipulations outside the incubator.

Both cooling and overheating during routine in vitro manipulation of oocytes and embryos can result in short- or long-term temperature shock [11]. The severity of these temperature shifts depends on the type of dish used, the accuracy and function of the warming device, volume of culture media and the presence of an oil overlay [12]. The negative consequences depend on both the duration and severity of suboptimal temperature exposure, as well as the developmental stage of the embryo [13]. Cells respond to changes in temperature by changing their metabolism. Cooling results in a slower metabolism [14].

A cellular structure highly susceptible to temperature fluctuations is the spindle apparatus. Microtubules represent the major component of the meiotic spindle, and these structures assemble and disassemble in response to changes in temperature [15–17]. Cooling and overheating of oocytes have been shown to affect the morphology of the meiotic spindle in a number of species, and even miniscule changes as small as 0.3 °C for very short times can cause irreversible damage [17, 18]. The meiotic spindle is a determinant of oocyte viability, responsible for correct chromosome alignment and segregation during metaphase I and metaphase II, genomic stability and consequently fertilisation and post-fertilisation events [19, 20]. More than 80% of aneuploidies originating from fragmented human embryos following ART originate from the oocyte [12, 21]. The correlation between meiotic spindle and oocyte quality is important to remember, as poor spindle architecture can result in high levels of aneuploidy, which in turn could result in embryo degeneration, spontaneous abortion and disease [19, 20, 22]. A recent study showed oocytes with normal spindle morphology were more likely to generate a euploid embryo, compared to oocytes with an abnormal spindle when viewed by polarised light microscopy [23]. The morphology of the meiotic spindle has also been associated with improved fertilisation rate, pronuclear formation, early cleavage, embryo developmental competence and blastulation rates, although using spindle morphology alone cannot predict the success of an ART cycle [24, 25].

Changes in temperature during oocyte and embryo development can influence pre-implantation developmental processes causing alterations in methylation patterns of imprinted genes resulting in developmental failure and mortality [26–28]. Moreover, heat shock in embryos can disrupt protein synthesis, cytoskeleton organisation and zona pellucida architecture resulting in developmental delays and embryo arrest [27]. Considering all the above-mentioned outcomes, stringent control of temperature throughout the in vitro period is an absolute necessity.

7.2.1.1 Control of Temperature as a Variable

Incubation Temperature

The incubator and culture system act as a substitute reproductive tract during the in vitro period. Controlling temperature for this period is dependent on the incubator. Historically, cell culture incubators were used for embryo culture; however, over time, incubator technology has been refined to meet the precise demands of a constant environment. A shortfall of traditional 'big-box' incubators has been having one large door, with frequent openings causing fluctuations in temperature. The modern big-box incubators are now fitted with multiple smaller doors, minimising the impact of openings. More refined design of 'benchtop' incubators, including multiple smaller chambers, has led to an even greater control over temperature. Comparison of these two types of incubators has shown the benchtop incubator to have a decreased recovery time of temperature resulting in a significantly higher blastocyst yield [29, 30]. A major shortcoming of benchtop incubators is that temperature sensors are located in few parts of the incubator, leading to an uneven distribution of heat (hot spots and cold spots) in contrast to big-box incubators that have the sensor inside the chamber and use a fan for even heat distribution. Another important consideration is minimisation of incubator door openings. Using working incubators for intermediate holding of dishes during processes that require frequent access to the sample can achieve optimal chamber temperature stability.

Whichever incubator system is used, controlling incubator temperature first requires comprehensive temperature mapping. Using an accurate thermometer (±0.2 °C) mapping should be done in all compartments of the incubator by measuring temperature within the culture system and the chamber air at each mapping point. The validation report should contain the culture temperature for each mapping position, together with the corresponding air temperature (±SD). Temperature is thereafter monitored daily using an independent accurate device (±0.2 °C). Noting the display temperature daily does not provide any control over culture temperature. Modern laboratories are equipped with continuous temperature monitoring systems allowing computer-logged 24-h monitoring with built-in alarm systems. Established processes are needed to include the appropriate action to be taken if the temperature falls outside the set range (i.e. ±0.2 °C).

Temperature of Warming Devices

Cooling is more likely to occur when manipulating oocytes and embryos outside the incubator. Warming devices need to be validated through temperature mapping in the same way as incubators to assure a stable temperature of 37 ± 0.2 °C is maintained. For tube warmers, the temperature is validated in a tube containing fluid in each position of the warmer. Testing each culture device separately is important as plasticware have different designs and distance between the actual bottom of the dish and the heated stage. There are dishes available for embryo culture where the plastic is in direct contact with the heated stage (Vitrolife). Surface temperature at each mapping point is also measured. The validation report should contain the culture temperature for each mapping position together with the corresponding surface temperature (±SD). The temperature for the warming device is to be adjusted to assure 37 ± 0.2 °C inside the culture system itself. Through full mapping/validation, the laboratory can identify possible hot/cold spots in order to avoid this affecting gametes and embryos. Based on the validation, the limits for the measurement can also be established. Following mapping, target temperatures are verified by daily measurements using a surface probe for the areas marked as appropriate for use.

When considering controlling temperature variations during manipulations, it is also important that the embryologists understand that the process itself can introduce fluctuations. Insertion and swirling of a glass pipette into the media during the process of denudation for ICSI on a temperature-validated heated stage has been shown to result in rapid cooling with different methodologies also resulting in alternate cooling rates [31]. This highlights the importance of validating different methods.

Room/Laboratory Temperature

Laboratory temperature needs to be controlled and kept constant. Laboratory equipment is designed to operate at the standard laboratory temperature of 22–24 ± 2 °C, and fluctuations in room temperature (RT) will affect equipment performance and results [32]. A dish taken out of the incubator and placed on a heated stage at RT of 20 °C will experience different cooling rates than a laboratory where the temperature is held at 30 °C. As a result, core processes such as ICSI and vitrification will be indirectly influenced. Although high laboratory temperatures >26 °C could be considered beneficial for reducing cooling rates when embryos are handled outside the incubator, high temperatures may result in increased risks for incubator overheating, staff discomfort and bacterial and fungal contamination.

Laboratory temperature needs to be monitored daily, preferably at more than one point of measurement in the room. All storage areas need to be included in the monitoring scheme, as extreme temperatures will affect the toxicity and cause plasticiser built-up in plasticware and other plastic devices. Monitoring should be done using a thermometer with an accuracy of ±2 °C and a process in place for appropriate action if temperature falls outside the set range of 22–24 ± 2 °C.

Refrigerator/Freezer Temperatures

The temperature of stored culture media and reagents is important for product stability and quality. The manufacturer specifies the accurate storage temperature range on the package insert. Similarly, the model of refrigerator needs to comply with storage requirements stipulated by the manufacturer. For storing culture media, pharmaceutical refrigerators fitted with a circulation fan and wire shelves to allow for even distribution of temperature throughout the chamber are used. It is important to not over-stack shelves and to place the

products in a manner that does not disturb the flow of cold air in the refrigerator. Many laboratories store culture media in a standard kitchen refrigerator. This risks compromising the quality and stability of the media as these refrigerators do not maintain accurate temperature throughout the chamber and would normally have a 2–3 °C difference between the top and the bottom shelves.

The temperature inside the refrigerator/freezer is set according to the manufacturer's specifications and needs to be monitored at least once daily using a thermometer with an accuracy agreeing with the limitations (i.e. ±1 °C) accompanied with recording and actions to take if a reading is found to be outside the set range. As for incubators, continuous 24-h monitoring of refrigerator temperature is recommended.

7

7.2.2 pH

Maintaining a constant pH in the culture environment is a critical aspect of an IVF laboratory and has significant effects on the outcomes [2, 33]. The regulation of intracellular pH (pHi) is essential for maintaining cell homeostasis in oocytes and embryos [34]. Controlling the pHi is crucial for a range of cellular processes such as metabolism, DNA and protein synthesis, respiration, enzyme activity, cell differentiation, growth and proliferation, membrane transport, cell-cell communication, calcium level modulation and cytoskeletal dynamics [5, 35, 36].

The pHi of human embryos ranges between 7.1 and 7.2 [37]. While most cells have a wide range of mechanisms to regulate pHi, early embryos have relatively little homeostatic capability [38]. In early embryos, pHi is regulated using three transporters: Na^+/H^+ antiporter, $Na^+/ HCO3^-/Cl^-$ transporter and the $HCO3^-/Cl^-$ transporter [39]. The Na^+/H^+ antiporter is functional under acidic conditions (pH 6.8), actively removing protons from the cytosol in exchange for extracellular sodium ions. The $Na^+/ HCO3^-/Cl^-$ transporter becomes active at more neutral pH ranging from 6.8 to 7.1 and uses bicarbonate ions to increase pHi during an acidic load. The $HCO3^-/Cl^-$ exchanger is active at slightly more alkaline conditions of 7.2–7.3 and transports bicarbonate ions from the cytosol in exchange for chloride ions. With changing pHi, the transporter system exponentially increases in activity until the pHi returns to normal [35].

The sequential changes of the reproductive tract, with alkaline milieu of the oviduct to the more acidic conditions of the uterus along with the differential activity and expression of transporters across pre-implantation development, underline the susceptibility of embryos to changes in pH in vitro where conditions are not comparable to those in vivo. Although cleavage embryos grown in vivo can effectively regulate small pH changes, in vitro culture exposes embryos to rapid fluctuation patterns of pH by incubator door openings and observations of embryos that result in reduced development potential [40].

In somatic cells, minute alterations in pHi can result in disruption of a number of crucial processes such as respiration, protein synthesis and nucleic acid synthesis [35]. In comparison, somatic cells are relatively stable, while embryos are dynamic, undergoing several rounds of cleavage, genome activation, compaction and blastulation over 120 h. Taking this into account, it is unsurprising that even the smallest fluctuations in pH can be catastrophic.

Exposing murine embryos to the weak acids, such as pyruvate and lactate, has been shown to decrease the pHi to sub-physiological levels that results in developmental arrest at the two-cell stage and blastulation inhibition [41]. In the same study, it was also shown that acidic conditions perturb metabolism, another sign of embryo stress [41]. In a subsequent study conducted using hamster embryos, exposure to weak acids and bases resulted in the disruption of mitochondrial organisation within the cytoplasm with the effect being greater after intracellular alkalinisation [42]. It is evident therefore that embryos are extremely susceptible to pH changes, and this can cause irreversible damage. Other than the pHi, embryos are exposed to the endogenous external media pH (pHe). While it has been suggested that embryos can tolerate some fluctuation in pHe, recovery of pHi is mostly irreversible [7, 42, 43].

7.2.2.1 Control of pH as a Variable

Control of Incubator pH

The pHe is regulated in vivo by a bicarbonate-buffered system via carbonic anhydrase and circulating CO_2, whilst in vitro, the pH of culture media (pHe) is regulated by the level of CO_2 in the gassed atmosphere and the use of buffers in culture media. These buffers are used to accommodate the minute pH fluctuations caused by the accumulation and depletion of other alkaline and acidic substances in the media such as amino acids. Commonly the pH of embryo culture media ranges from pH 7.2 to 7.4, allowing the embryo to maintain a pHi of 7.1–7.2. Bicarbonate, HEPES and MOPS buffers all have buffer capacity in these ranges and are commonly used in embryo culture and handling medium.

The pH scale is logarithmic; a 0.2 variation in pHe represents a 60% change in H^+ concentration. It is therefore crucial to minimise pHe fluctuations in order to reduce variability in the culture system. Factors including temperature, humidity, altitude, incubator gas concentration as well as handling will affect the pHe [39]. The bicarbonate buffering system is the most physiological and commonly used system in embryo culture. However, this system is dependent on the concentration of CO_2 in the surrounding atmosphere. Most commercial embryo culture media have a bicarbonate concentration requiring a CO_2 concentration between 5 and 6% to maintain a pH range of 7.2–7.4. If a bicarbonate-buffered medium is taken out of the gassed incubator and is exposed to ambient CO_2 concentrations (0.04%), the pH begins to rise dramatically within minutes and can take up to 30 min for the pH to decrease to the desired levels when returned to the incubator [5].

The pH inside the incubator needs to be monitored with specialised pH meters using the same conditions including

volume of media and/or oil overlay, which gametes and embryos are exposed to during culture. Alternatively, employing a blood gas analyser will result in more accurate measurements. The manufacturer sets the pH, and its accuracy should be within ±0.2 units. The pH measurements need to be undertaken on a weekly basis and all measurements recorded in a logbook with actions outlined if measurements fall outside the specified range. In addition, pH measurements are considered essential every time a new lot of culture media is introduced and also when a new gas cylinder is installed.

Control of pH Outside the Incubator

Since exposure of embryos to alkaline conditions is unacceptable for culturing embryos, handling procedures outside the incubator requires appropriate buffering systems. HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) is a highly stable zwitterion with a midpoint buffering range (pKa) of 7.31 at 37 °C, highly soluble in water, with low permeability through plasma membranes making it ideal for use in cell culture [44]. MOPS buffer (3-(*N*-morpholino)propanesulfonic acid) is another zwitterion with similar properties to HEPES and a 7.04 pKa at 37 °C [44]. The main advantage of these buffers is that they are not dependent on CO_2 to maintain a stable pH making them suitable as buffers in handling media for benchtop procedures such as oocyte collection, ICSI and embryo biopsy.

Several reports have found that HEPES may be negatively impacting embryo development [45–47]. Consequently, some manufacturers have replaced HEPES with MOPS in handling media. Despite this, there is limited data on the effects of MOPS on embryo growth development [48]. MOPS has been shown to support in vitro maturation of murine oocytes and bovine embryo development just as well as HEPES [49, 50]. Furthermore, even though gene expression of environmentally sensitive genes differs from in vivo derived embryos, the two buffers showed no significant difference between them [50].

Buffer capacity can have a significant impact on gamete and embryo quality [51]. Nonetheless, it is important to critically assess the literature and scrutinise culture conditions. Some studies, for example, have used HEPES or MOPS as fertilisation media to assess developmental competence of embryos [47, 49]. However, the time and duration of exposure to HEPES or MOPS used in these studies do not replicate the processes in a clinical setting where the time in HEPES- and MOPS-buffered medium is kept at a minimum [45, 50]. With the exception of ICSI, which can take a few minutes, there are no long-term cultures.

An alternative approach to HEPES or MOPS is to handle gametes and embryos in bicarbonate-buffered media supplemented with phenol red as a pH indicator. This compound can be used in concentrations from 0.003 to 0.010 g/L, and when the pH falls outside the range of 7.15–7.35, its pink-rose colour changes. In particular, at a pH <7.15, the colour becomes yellow, and at pH >7.45, the colour becomes red-purple. Some reports however have shown that phenol red has a mild oestrogenic effect and can be toxic [52, 53]. In a different study, it was shown that at such low concentrations, it is impossible for phenol red to induce estrogenic effects [54]. Nevertheless, the possible toxicity and the inability to quantify pH changes when using it make phenol red not ideal for use.

Closed Workstations

The use of closed workstations that control temperature and gas can also be an alternative to the use of HEPES- and MOPS-buffered media. There is a range of workstation options with portable models, models with the possibility of O_2 control and models with fittings for stereomicroscope and inverted microscopes. The shortcoming of using such workstations is that the aseptic conditions are somehow lost, because of the turbulent flow inside the workstation. Furthermore, there is an increased risk of bacterial and fungal growth and the matter of decontamination and cleaning in situations of spillage where fumes from methanol would be re-circulating inside the workstation.

In the past few years, introduction of time-lapse incubators has enabled the continuous surveillance for the morphological evaluation of in vitro–derived embryos. This offers the benefits of uninterrupted culture, flexible workflow and improved embryo selection since more information is gained for the relationship between development and timing [55]. Furthermore, correlation has been drawn between time-lapse parameters and developmental competence, aneuploidy and pregnancy [56–58]. Nonetheless, it is well documented that good morphology embryos may well be aneuploidy, and therefore aneuploidy screening is still considered as the major selection tool.

7.2.3 Osmolarity

The osmolarity of culture media is critical when considering the optimum media for culture of pre-implantation embryos. The osmolarity of the female reproductive tract ranges between 310 and 370 mOsmol/kg for most species [43, 59]. The osmolarity of the human uterine fluid is in the range of 276–307 mOsmol/kg in unstimulated women and 305 mOsmol/kg in stimulated cycles [60]. Original attempts to culture embryos at osmolarity levels of over 300 mOsmol/kg have proven fruitless, with studies showing that mouse zygotes fail to develop in vitro at these levels. Specific amino acids have been suggested to serve as protective osmolytes allowing survival of in vivo embryos at high physiological osmolarity [61–63]. The optimal osmolarity supporting the growth and development of embryos in vitro varies among species with most modern culture media having an osmolarity of 260 mOsmol/kg [64–66].

Embryo culture at suboptimal osmolarity has been shown to affect embryo developmental competence and increase expression of apoptosis-related genes [59]. The effect of suboptimal osmolarity is suggested to be stage specific with mouse oocytes and one-cell embryos being more susceptible

to osmotic stress than cleavage stage and post compaction embryos [67]. Mammalian pre-implantation embryos employ a range of mechanisms to adjust cell volume in response to osmolarity changes [64]. When adjusting to changes in osmolarity, most cells will alter their volume by regulating osmotic pressure across their membranes and alter the concentration of osmotically active osmolytes in the cytoplasm [68]. Solute releasing reverses cell swelling, while shrinkage is counteracted by the active import of solutes [62]. In hypertonic conditions, the embryo increases its intracellular concentration of inorganic ions (Na^+, K^+ and Cl^+) resulting in an increased osmotic pressure. This leads to cellular proteins being destabilised and a disruption in metabolism [69]. The presence of organic osmolytes in media with high osmolarity is therefore necessary to maintain cell volume and counteract for the deleterious effects of intracellular inorganic ions. Modern culture media utilise organic osmolytes such as methyl ammonium compounds (e.g. betaine), small zwitterionic amino acids (e.g. glycine and taurine) or sugars and polyols (e.g. myo-inositol) [62].

7.2.3.1 Control of Osmolarity

Studies of evaporation in culture media have shown that the surface area can affect osmolarity [70]. A large dish surface with a small volume of media can increase the osmolarity value, whilst an increase in volume leads to osmolarity stabilisation [71]. The volume of culture media with proper ratio to surface area is necessary to achieve a stable osmolarity. As it has been shown previously, when a dish containing 3.0 ml of culture media is left at RT for 30 min, it causes an osmolarity rise of 5%, whilst when the dish is left at RT for an hour, osmolarity rises by 14% [72]. Dishes that are left for extended time without oil overlay should be checked for possible evaporation and increase of osmolarity.

When culture dishes are placed in a gassed incubator, airflow on the liquid will cause an increase of evaporation even in a humidified incubator, and that will inevitably cause an increase in osmolarity. An effective way to reduce evaporation of the culture media and osmolarity changes is to overlay culture drops with mineral oil. Making culture dishes should be done at room temperature with medium that is taken out of the refrigerator just before dish preparation. Dish preparation is done on a non-heated surface area of the laminar airflow hood, and under no circumstances should the heated surface be used. Having dishes with a volume of <20 μl culture media when using oil overlay should be avoided. A further factor to be considered is the humidity of the incubator. It is vital to maintain the optimum humidity in the incubator in order to keep evaporation of the media to a minimum and maintain a stable osmolarity.

7.2.4 Culture Media

Although the necessities of embryos during pre-implantation development have not been fully described, ongoing research has been able to establish the complexity of needs during different stages of development [73]. As an outcome, media formulations have become more composite [74]. The success of an IVF program relies on an optimal culture system that reduces stress and ensures gamete and embryo viability and development. Up to the stage of embryonic genome activation, embryos are more susceptible to damage, since they have not developed their own mechanisms and solely rely on mRNA from the oocyte [75]. Even after embryonic genome activation, the culture environment can have a significant effect on embryonic transcription and translation affecting birth weight and infant health following IVF treatment [76, 77]. In addition, commercially supplied culture media and buffers can result in spontaneous formation of reactive oxygen species (ROS), as in the case of HEPES buffer, which can result in formation of hydrogen peroxide and disruption of signalling pathways associated with fertilisation [78]. In addition, the culture conditions have been shown to affect imprinting [79].

7.2.4.1 Control of Culture Media

Culture media is not a variable that can be controlled; however, selection is vital when it comes to the success of an IVF program. When commercial media are used, it is important to track batch numbers and composition of each batch. Other than the certificates of analyses that companies provide with information regarding their testing (mouse embryo assay, endotoxin testing, etc.), it is important to consider the manufacturer's quality control systems during production. Dataloggers can also be utilised to record temperatures of the culture media during transportation.

If homemade media are used, then the appropriate bioassays for testing have to be performed as defined by national and international regulations, whilst sperm survival toxicity tests can be employed for validation of media batches. Whatever the source of media, it is important to keep records of dates of use for each batch to allow for key performance indicators to be used effectively. When media are aliquoted in smaller volumes, it is vital that the aliquot containers are deemed appropriate for embryo culture and off-gassed for at least 24 h before use. Plasticware may contain Bisphenol-A, which has been shown to affect reproductive function [80]. Recording lot number of media versus aliquoting media in smaller volumes is preferable because it will minimise variability and provide better control of potential issues with other variables that might affect the success of the IVF program. All culture dishes should be pre-equilibrated for temperature and pH by being placed into the incubators a few hours before being used. Dish making has to be done in a sterile and efficient way. When making culture dishes by overlaying micro-drops by oil, no more than one dish can be made at a time to avoid changes in evaporation and osmolarity.

7.2.5 Oxygen Tension and Gas Supply

Oxygen is one of the most characterised elements that controls embryo function with its concentration in the female tract around 5% and atmospheric oxygen around 21% [81].

Oxygen can be converted to superoxide radicals that are deleterious to cells, and culture in atmospheric oxygen has been shown to affect transcription, translation, metabolism as well as cause epigenetic changes [82–85]. In addition, embryos cultured in atmospheric oxygen have been shown to be susceptible to ammonium build-up in the surrounding media [86].

In light of the stressors associated with the use of atmospheric oxygen, concerns have been raised about the rationale behind culturing embryos to the blastocyst stage [87]. On the other hand, the benefits of low oxygen culture (5–7%) have been documented in terms of embryo development and live birth rates, suggesting that utilising a low incubator oxygen tension is a more rational approach [88, 89]. Nevertheless, a number of clinics and countries still utilise atmospheric oxygen tension regardless of its negative effects. Instead of considering extended culture as a potentially harmful procedure, the birth rates derived following blastocyst transfers and vitrification need to be interpreted based on the culture conditions, with 20% oxygen levels considered unacceptable [90].

7.2.5.1 Control of Gas Supply

Embryo culture requires a reliable and consistent quality supply of gases and gas mixtures. All gases utilised in gamete and embryo culture have to be attained from suppliers that comply with international standards in terms of purity and specification. In all the gases for the 'atmospheric' mixture of 6% CO_2 in air or the 'physiological' mixture of 6% CO_2, 5–7% O_2 in N_2 have to be of high purity. The low oxygen environment can be created either by supplying the incubator with pre-mixed gas or by suppressing the atmospheric O_2 using N_2. Auto-changeover manifolds have to be in place to ensure constant supply of gas into the incubator systems. Gas cylinders are supplied with certificate of analysis and the incubator CO_2 and O_2 levels monitored daily and actions taken if readings fall outside the range.

The pH can be disturbed by the gas quality. Therefore, each time a new cylinder is installed, the pH has to be measured. This is particularly important in certain countries, where either gas cylinders are not certified or the gas levels can vary even with certification. A blood gas analyser is a good tool to be employed for verification of CO_2 and O_2 levels along with pH readings. When the pH of an incubator falls out of the specified range, the CO_2 level is adjusted accordingly. Specifically, due to the inverse relationship of CO_2 and pH, lowering CO_2 levels can be used to raise pH, while raising CO_2 levels can be used to lower pH until the levels reach the pre-determined acceptable range. There is a range of gas-measuring devices including devices with thermal conductivity sensors, devices with infrared sensors and portable devices that can simultaneously measure CO_2, O_2, relative humidity and temperature. Before using such a device, calibration against a standard certified gas is essential.

7.2.6 Air Quality

In order to achieve stable results, the laboratory environment needs to be tightly controlled and a vital component is air cleanliness. In relation to the air quality, the presence and concentration of volatile organic compounds (VOCs), airborne particles and growth of any contaminants such as bacteria and fungus will impact both the safety and success of embryo culture [91, 92]. The environment inside the IVF laboratory can contain a number of microorganisms that are indigenous to humans [93]. Airborne particles vary in size, and their concentration can increase with personnel movement, operation of equipment as well as infection. VOCs are synthetic chemicals that can be vaporised; these include paints, pesticides, glue, alcohol and cleaning products. Exposure of human embryos to aldehydes and motor vehicle emissions has been shown to reduce pregnancy rates [94].

7.2.6.1 Control of Air Quality

Clean air means control of ventilation, filtration and adoption of aseptic processes, along with cleaning, disinfection and maintenance of equipment [95]. The position and number of air exchanges of the heating, ventilation and air conditioning (HVAC) system are important in order to reduce the number of particle counts. Air quality can be maintained by having positive pressure in the laboratory with a differential of 10–15 Pascals from the adjacent areas. In the embryology laboratory, when the gametes and embryos are exposed to environmental conditions outside the incubator, laminar airflow hoods and biological safety cabinets are used. The use of such flow hoods and cabinets are essential since they clean the air by the use of high-efficiency particulate air (HEPA) filters that capture particles of 5 μm or bigger, while UV light or heat can also be used. On the other hand, HEPA filters cannot trap VOCs that are smaller in size [96].

When gametes and embryos are placed in the incubator, the use of VOC filters in the incubator instead of HEPA filters has been shown to produce higher blastocyst rates [2]. Furthermore, the use of solid carbon and potassium permanganate filters has also been shown effective in removing VOCs [91]. These systems are available for gas bottle inlets or are installed within the incubator while they can also be integrated in the central air conditioning system of the laboratory or purchased as standalone units. The efficiency of these filters should be monitored on a regular basis.

Active measures need to be taken to minimise particle counts and VOCs. Particular attention should be given to the embryology laboratory design. Furnishings such as Formica, pressed wood or fireboard release formaldehyde, and hence, their use is prohibited. Flooring should also be carefully considered and the use of glue avoided. As it concerns paint, water-based paints with low VOC potential should be selected with the use of large industrial construction fans and venting of the exhaust to the exterior. All plasticware should be out-gassed by being unpacked from the plastic wrapping in order to release accumulated VOCs before use. All cotton

and other high-lint materials should be banned from the laboratory with preference towards low-lint laboratory clothing. To ensure standardised air quality, monitoring should include the measurement of VOCs and particle counts in operation and at rest. To achieve clean room standards, these parameters need to be measured daily and recorded with appropriate action described should a measurement fall outside the safe range.

Anything that enters the embryology laboratory including consumables, equipment and fresh, frozen and imported gametes and embryos can be a source of contamination. Even when embryologists apply stringent aseptic techniques, there are still contamination risks if the environment is not controlled. To reduce chances of infection, the laboratory should always be tidy and clean with the personnel maintaining high hygiene standards and good waste disposal management. Furthermore, access to the laboratory should only be permitted to authorised personnel and a routine for daily cleaning and decontamination should be established. All surfaces must be cleaned with 70% ethanol or commercially available embryo-tested spray, followed by rinsing with sterile water to remove any toxic overdue from the disinfectants.

In order to detect environmental microorganisms such as bacteria and fungi, Sabouraud dextrose agar (SDA) and tryptic soy agar (TSA) settle plates can be used and placed on work surfaces [97]. In addition, swabs should be used for hard-to-reach and non-flat surfaces. Active air sampling of viable particles is also necessary for microbiological monitoring of the IVF laboratory [95]. Active sampling is done by drawing air with the syringe and expelling it over the SDA and TSA plates. The selection and use of the sampling method should be validated in terms of its compatibility in the recovery and incubation conditions to allow growth and identification of microorganisms. These culture conditions vary, with bacteria requiring 2–5 days at 30–35 °C, while fungi and moulds require culture for 5–7 days at 20–25 °C [97]. The tests for the detection of microorganisms should be conducted every 3 months.

7.2.7 Light

Although it is generally assumed that light has no effect on gametes and embryos, in media containing HEPES buffer, it has been shown that ROS are produced with exposure to light [98]. Furthermore, cool white fluorescent light has been shown to produce more ROS in mouse zygotes, and embryos have been shown to have better developmental potential in the absence of light [99]. Light damages the embryos either directly by activating stress genes and damaging DNA by ionisation or indirectly by oxidation of components of culture media or oil [100].

7.2.7.1 Control of Light

The effect of light on zygotes and embryos therefore should not be ignored. Embryology laboratories should not be exposed to direct sunlight. Low-wavelength light energy (<500 nm) can be avoided by selecting appropriate light sources (avoiding fluorescent lighting) and/or use of filtration systems. Additionally, the exposure of gametes and embryos to light should be minimised by reducing the time they spent outside the incubator. Using a green filter when viewing and manipulating gametes and embryos under the microscope can be a practical solution. Caution is required when using hood lights and headlamps. Switching off the lights is the only way to minimise stress to the embryos.

7.2.8 Air Humidity

If the conditions in the laboratory vary between seasons or days of the week, then outcomes can be affected. This includes the humidity of the laboratory that should be set and maintained at levels below 50%. This is because contaminants such as fungi increase when humidity levels are high, with humidity levels between 40 and 60% shown to minimise fungal growth [101]. In addition, microorganism inactivation by UV radiation has been shown to be dependent upon relative air humidity [102]. The humidity in the ambient air can also cause formation of ice and frost on the stored cryopreserved samples located in vapour tanks, increasing the risk for contamination and also affecting the readability of labels and barcodes [103]. Air humidity levels should be checked and recorded on a daily basis.

7.2.9 Sound/Music Vibrations

One of the variables in the IVF laboratory that has not been extensively researched is the effect of sound and music vibrations on gamete and embryo development. The first study that conducted monitoring of early embryonic development of chicken eggs using acoustic resonance frequency analysis showed that fertile eggs decrease their resonant frequency at a certain stage of incubation [104]. A follow-up study monitoring the course of vibration parameters during early incubation of chicken eggs showed that the point of vibration alteration was correlated with the diameter of the eggs and prediction of hatching time [105]. In humans, application of a tilting embryo culture system was shown to improve embryo development to the blastocyst stage [106]. Similarly, micro-funnel culture conditions have shown improvement in embryo development and pregnancy rates [107]. The use of mechanical micro-vibrations to the culture system also showed improvement in pregnancy rates [108]. In a recent study, the effect of music as a source of mechanical vibrations was explored, and results showed that any type of music (pop, heavy metal, classical) played in the laboratory environment improved the fertilisation rates, but did not have an impact on embryo development as assessed on day 2 [109]. From the above-mentioned studies, it appears that dynamisation during culture can be beneficial in assisted reproduction; however, additional research is required.

7.2.10 Equipment Performance

The success of any IVF program relies heavily on the performance of the equipment it possesses. All equipment must operate within tolerable limits that are pre-set based on the values that are biologically optimal having in mind the instrument variability. For example, a type-K micro-probe will not be as accurate as using a Platinum Sensor PT100 for temperature monitoring because the type-K micro-probe can be affected by electromagnetic fields and lacks precision to detect differences of ±0.1 °C at 37 °C. Furthermore, newer technology equipment have built-in sensors and readings that can be reviewed on a daily basis.

An often-neglected task in the IVF laboratory is the performance of daily quality control for monitoring of equipment. To ensure that all equipment function within acceptable limits, daily readings are conducted either manually or through continuous monitoring systems. The data are either recorded in logbooks or transferred by a wireless system to the base if continuous monitoring systems are in place. The monitoring logbook should include measurement value, acceptable range and action taken if a measurement falls outside the given range, with each entry requiring a staff signature. The data are checked daily in comparison to the instrument display and weekly to verify that there are no fluctuations over time. In addition, alarm options can provide external control, where SMS messages, phone calls and alarm relays can be programmed if an instrument falls outside the specified acceptable limits.

A user's manual is required for all operational equipment alongside the appropriate validations. These validations include documentation that the equipment has been installed according to the requirements in a safe manner, along with documentation of its functionality according to specifications. Performance of the equipment must also be validated and documented by testing that equipment function within appropriate ranges and generate reproducible results. Equipment failure or underperformance can be minimised by preventative maintenance. Records for the calibration are kept together with maintenance records. Backup solutions must be in place for critical pieces of equipment such as incubators, aspiration pumps and inverted microscopes. The use of consumables and supplies should also be tightly monitored and controlled. All disposable items must be non-embryo-toxic with quality assurance certificates. Similarly to the culture media, lot numbers for plasticware need to be recorded when in use.

7.2.11 Staff Performance

The sophisticated process of ART requires coordinated teamwork and collaboration of a team of individuals with different personalities and characteristics working towards a common purpose. Like domino pieces, the success of an IVF program relies on the interdependence between the members of the team to deliver the end result. The ideal IVF laboratory will have a safe workplace with staff sufficiently trained with regular review of their performance. All staff should have training logbooks to provide records for the tasks that have demonstrated competency as assessed by senior staff.

Thereafter, competence assessments are recorded for all trained embryologists twice a year. Competency can also be obtained by the use of online quality assessment schedules such as UKNEQAS (► http://www.ukneqas.org.uk) and FertAid (► http://www.fertaid.com). Monitoring individual staff performance can be beneficial for individual embryologists, the embryology team and the results of the IVF centre. When the records show a lower key performance indicator (KPI) for a certain task, the individual can be assessed performing the task to be assessed and re-trained if this is deemed necessary. In that way, individual embryologists can improve their performance and effectiveness. It is important that this exercise is performed without criticism. In addition, members of the team can observe and learn from embryologists who perform well in certain tasks. In that way, all members of the team can entrust in each of their colleague's efficiency and professionalism in the pursuit of delivering the best treatment to patients. Ultimately, all members of the team are critical to success; therefore, even if one team member fails to proficiently complete a certain task, it can be reflected on the results of the IVF centre.

7.3 Final Summary

There are a number of variables influencing the successful outcome of an IVF cycle. The knowledge obtained over the years has led to a standardisation of processes that has allowed for the increase of embryo utilisation interwoven with a reduction in embryos transferred and superior pregnancy and live birth rates. Due to the multifactorial nature of ART, not everything falls under the control of the laboratory. Stimulation variables and patient dissimilarity in medication response can also directly affect results. However, the lack of rigorous control over variables will undoubtedly lead to failures with answers and solutions to problems not easily found. Controlling variables is therefore the key to success.

Review Questions

1. How can temperature variations be controlled during embryo culture?
2. How can temperature variations be minimised during manipulations outside the incubator?
3. What type of refrigerator is required for correct storage of culture medium?

4. What are the pros and cons of having a warm laboratory?
5. What is the optimal pH range for embryo culture?
6. How can fluctuations in pH be avoided?
7. How can the embryologist minimise changes to culture medium osmolarity?
8. What important environmental controls and tests are necessary to safeguard a constant environment of the laboratory?
9. How can traceability be achieved with regard to devices used in the laboratory?
10. What are the important components of equipment quality control?
11. What are the benefits of monitoring individual staff performance…?
    1. For the individual embryologist
    2. For the embryology team
    3. For the results of the IVF centre

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Daily, Weekly, and Regular Preparations for the IVF Laboratory

Klaus E. Wiemer

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Learning Objectives

- Provide an overview of how to establish standards of performance for various aspects of the embryology laboratory.
- Recognize that different aspects within the laboratory require different levels of measurements.
- Provide suggestions for daily, weekly, and regular measurements for important parameters as they pertain to different aspects of the IVF laboratory.

8.1 Introduction

In order to establish and maintain the highest standards of performance in an embryology laboratory, it is recommended that certain preparatory measures occur on a daily, weekly, and regular interval basis. Standards for variables that can impact embryonic development should be determined for each laboratory, recorded, and verified that values are acceptable. This would include taking and recording measurements of important variables such as heated surface temperatures, pH values of culture media, and liquid nitrogen levels in cryostorage tanks. In addition, standards should be established for the proper maintenance of equipment and normal ranges of variation permitted within the laboratory. These standards should be established by determining the ideal ranges of acceptable variability through empirical tests designed to optimize the laboratory environment for growing healthy embryos.

In an effort to optimize use of laboratory space, increase efficiency of workflow, and accommodate staff effectively within the laboratory, the laboratory should be designed so that different embryology procedures are performed in different areas that are designated for those particular procedures [1]. For example, the laboratory can be divided into three to four separate rooms based upon primary function, such as cryopreservation/cryostorage room, main embryology laboratory, a room for performing embryo biopsies as well as cell preparation, and a room for embryo culture dish preparation. A separate room for andrology-related procedures is a viable option if space permits. Having a separate room for semen analysis and sperm preparations is ideal since this lab space is often much busier and the potential for contamination from sperm samples is greater. The andrology laboratory also houses all the equipment used for various types of diagnostic testing, so traffic and probability of introducing potentially infectious material from a variety of sources are higher.

The main laboratory should serve as designated space where all general embryology procedures take place and would house the majority of lab equipment. With increasing use of embryo biopsy, having a room or space that is not located in the main traffic of the embryology lab is ideal. This allows a quiet space for embryo biopsy and processing cells resulting from the biopsy procedure. Embryo biopsy and cell preparation require high levels of concentration and constant communication with the witness who is helping with the cases. Less interruptions and distractions are an ideal setting and reduce the potential for errors. The media preparation room is designed specifically for refrigeration of media, preparation of culture media, the weighing of chemicals, and general laboratory preparations. In the media preparation room, having a sterile hood to prepare dishes and material for embryo culture is ideal as well. The cryopreservation/cryostorage room is utilized for gamete/embryo freezing, as well as short- or long-term cryostorage of embryos and oocytes. Having bench or hood space to house dissecting microscopes is optimal for freezing and thawing. With the different thawing protocols now used for vitrified samples, having a dissecting scope with a heated stage adjacent to a scope with a non-heated surface is ideal because thaw protocols often call for initial thawing at 37 °C with the subsequent steps being performed at room temperature.

In addition to these three to four main areas of the embryology laboratory, two other areas of high importance are the gas manifold room and HVAC room. This chapter is organized based on the daily, weekly, and regular preparations required in these different sections of the clinical embryology laboratory. Establishment of standards will be discussed as well.

8.2 Cryopreservation/Cryostorage Room

The advantage of having a separate cryopreservation room is that oocyte and/or embryo cryopreservation procedures can be performed concurrently, while general embryology procedures are performed in the main embryology laboratory. Therefore, general workflow is not interrupted by any cryopreservation procedures. This allows cryopreservation to occur without having to allow hoods designated for general embryology to cool down, for example. The cryopreservation room also provides a designated area for cryostorage tanks for short- and/or long-term cryostorage of embryos and oocytes. The cryopreservation/cryostorage room should be designed so that it contains a liquid nitrogen outlet, space for cryostorage tanks, and a laminar flow hood for performing cryopreservation-related functions such as dish preparation for vitrification and/or thawing.

As mentioned in the introduction, vitrification has become the cryopreservation method of choice. For many thawing protocols, dissecting microscopes with heated stages and ovens to house warming solutions are now required for the initial thawing step. If this is the case, then temperatures of these heated devices should be checked every day. Temperature values should be established to ensure the media is at the proper thaw temperature. For convenience purposes, the placement of incubators to house specimens for cryopreservation is becoming more commonplace. If this is the case, then incubator temperatures, gas levels, and water levels should be checked on a regular basis as well.

It is advantageous to have liquid nitrogen access in the cryopreservation lab for various reasons. Some of these reasons include the ease with which cryostorage tanks can be

refilled, liquid nitrogen can be procured for use during freezing/thawing procedures, and liquid nitrogen can easily be obtained for temporary removal of cryostorage canes from storage tanks during cryotank inventory. Having liquid nitrogen access in the freezing lab can also prove to be invaluable during emergencies as samples are trying to be saved or moved to other tanks. Having access to liquid nitrogen in the lab also reduces the risk of introducing contaminants in the lab by not having to leave the clean area to enter an often more dirty room like the tank room where liquid nitrogen is often stored.

A number of measurements should be performed in the cryopreservation/cryostorage room on a daily, weekly, and regular interval basis. The following items should be checked and recorded on a daily basis: room temperature and temperatures of any refrigerators or freezers if applicable. In addition, liquid nitrogen levels in cryotanks, local alarms on cryotanks, and visual inspection of wires and temperature probes should also be performed on a daily basis. If any frozen material on the temperature probes is observed, the probes should be thawed and cleaned. We have found that checking the cryotanks on a daily basis helps us determine the integrity of the tank and rate of liquid nitrogen evaporation. Our experience is that self-contained rolling cryotanks typically lose about 1–2 cm per day in our lab environment along with normal tank openings. We have noticed that tanks that begin to lose more than that might be on the verge of losing their integrity. For example, tanks that all of a sudden start to drop 4–5 cm per day either have a faulty lid or the seal on the tank is in danger of breaking. Samples should be moved immediately until the source of the problem is located.

On a weekly basis, cryotanks should be refilled to their established levels. On a regular basis, integrity of all alarms hooked up to the cryotanks should be tested in order to ensure that the alarm will alert all lab personnel in case of a tank failure or drop in liquid nitrogen levels below established minimums. The call-back system on the alarm should be tested on a regular basis as well.

Standards should be established for filling cryotanks. If individual rolling cryotanks are used, minimum standards for liquid nitrogen levels in cryotanks must be developed in order to ensure that all cryopreserved embryos remain submerged [1]. Based upon the average amount of liquid nitrogen used by the laboratory, a regular schedule of liquid nitrogen delivery should be established. Clinics should determine if having a backup liquid nitrogen tank is warranted. A brief summary of recommended variables that should be measured with suggested time intervals is found in Table 8.1.

Table 8.1 Schedule of measurements performed within the cryopreservation/cryostorage lab

	Variable frequency	
Daily	**Weekly**	**Regularly**
Room temps	Fill tanks	Clean alarm probes
LN_2 levels	Check alarm integrity	Test alarm
Refrig-freezer temp		
Incubators/oven		
Heated surfaces		

A variable that can affect establishment of standards and requirements within the cryopreservation/cryostorage area is the type of cryostorage tanks used for holding cryopreserved specimens. IVF clinics that prefer to have their patients' embryos and oocytes shipped out for long-term storage after some period of time will most likely find that individual cryotanks are most appropriate for this course of action. A disadvantage of having individual rolling cryotanks is the frequency with which they must be filled in order to keep the liquid nitrogen levels in the tank at the proper level. Individual cryotanks also store fewer specimens than large auto-fill cryotanks. However, these tanks on a whole are very efficient at conserving liquid nitrogen and are considerably less expensive than auto-fill systems.

Clinics that elect to hold their patients' cryopreserved specimens in long-term storage "in house" will most likely find that large auto-fill cryotanks are most appropriate. Large auto-fill tanks can hold hundreds of specimens and are equipped with sensors that will automatically fill the tank with liquid nitrogen when it is necessary. Possible disadvantages of large auto-fill cryotanks include their cost and amount of space they occupy in the laboratory. Another potential disadvantage is that maintenance and repairs can be costly. If a lab decides to use these types of cryotanks, it is important that the lab purchase a tank that is designed to open frequently so that the seal on the lid will not wear out prematurely. Many of these tanks are not designed to be open on a daily basis.

It is important to establish a policy regarding shipper tanks as well. Standards should be established to ensure that transport tanks are filled to capacity with liquid nitrogen for 2 days prior to use. In most instances, the specifications on a particular brand of shipper tanks will state the weight of a tank when it is fully charged. Once a transport tank is charged, all liquid nitrogen can be poured out of the tank, and the canes containing the specimens can be placed into the charged tank and will be kept safely for 7–10 days.

8.3 Main Lab

The advantage of having a designated area for performing general embryology procedures is that it allows increased efficiency and speed of workflow within the laboratory. The close proximity of incubators, microscopes, and heated laminar flow hoods (or isolettes) is ideal for performing all

embryology procedures at optimal speed. In addition, the main laboratory should be designed so that the highest air pressure exists in this main room [2]. The main laboratory houses the majority of equipment found in an embryology laboratory. This includes laminar flow hoods, dissecting microscopes, isolettes, anti-vibration tables, inverted light microscopes with and without micromanipulation capabilities, audiovisual equipment, and incubators. As previously mentioned in the introduction, embryo biopsy cases might be performed in a separate room or alcoves within the main embryology lab.

A number of checks should be performed in the main laboratory on a daily, weekly, and regular interval basis. On a daily basis, the surface temperatures of heated microscope stages and hood surfaces should be checked and recorded, as well as room temperature and humidity. Surface temperatures of heated hood surfaces or isolettes should be taken in places where gametes are commonly kept. Temperature of block heaters should be noted as well on a daily basis. Incubator settings that should be measured on a daily basis include temperatures and gas levels within the incubators, pH of culture media, and status of water pan if applicable. With commonly used benchtop incubators, surface temperatures in both chambers, gas flow rates, and visualization/confirmation of humidification system should be noted on a daily basis. Ensuring good seals and proper gas flow is of paramount importance with these new benchtop incubators to confirm proper pH values for the embryo culture conditions.

Also on a daily basis, one should be certain that the values of the aforementioned items are within the laboratory's established range and adjustments are made as necessary. All temperature readings should be taken with a calibrated thermometer or thermocouple to ensure accuracy. If applicable, gas levels associated with the anti-vibration tables should be checked as well. Biohazardous materials and regular trash should be removed. Daily removal of all biohazardous materials reduces the risk of transmission of blood-borne pathogens and any possible airborne pathogens arising from the biohazardous material.

An important daily practice is unpacking new plasticware at the end of the day after all embryos are properly put away into incubators. Newly unpackaged plasticware should remain on shelving inside the back of a laminar flow hood to "off-gas" for a minimum of 48–72 h prior to use for any embryology procedure. The reason for this practice is to reduce potential exposure of gametes to VOCs from freshly unwrapped plasticware [3].

The following miscellaneous tasks should be performed on a weekly basis: refill liquid dispensers, change water in incubator pans, change water in humidification flasks used for benchtop incubators, check the water level of the circulating water baths that heat the surface of laminar flow hoods as well as the water level in heated isolettes, check the level of water in the tubes that thermometers sit in within incubators, change out glassware and hood accessories, remove discarded material from incubators, dispose of filled sharps containers, and restock all disposable materials in the laboratory.

It is highly recommended to evaluate the pH of the embryo culture media at least every week if not daily. The pH of the culture media should be evaluated in order to compare it with the CO_2 level readings obtained using a gas analyzer as well as on the incubator readout. CO_2 levels of the culture incubators should then be adjusted based on the pH values obtained using a high-quality pH meter. In addition to weekly pH evaluation of culture media, it is also recommended that a pH evaluation be performed on every new lot number of culture media that is received by the laboratory [1]. Weekly evaluation of the pH of culture media serves to elucidate the true levels of gases within the incubator as well as test the pH of new media before embryos are introduced to the new lot of culture media. For benchtop incubators, pH testing should be performed several times a week to ensure the seals on the incubators are intact. Often, the only way to tell that the seal on these incubators is starting to fail is by pH testing. If applicable, O_2 levels should also be determined and adjusted if outside of the established laboratory ranges.

A number of other tasks should be performed on a regular basis within the main area of the laboratory. These tasks include confirming that the uninterrupted power source (UPS) modules are working properly, checking the alarms and call-back system on all incubators, having maintenance performed on all microscopes, cleaning of all incubators, and changing all gas lines or incubator carbon filters if applicable. Water reservoirs in isolettes should be checked and cleaned as these reservoirs can be a major source of contamination. Laboratory floors and surface areas should be cleaned on a regular basis as well. A summary of items that should be checked on a regular basis can be found in Table 8.2.

Standards should be established and applied to equipment as well as procedures performed within the main area of the embryology laboratory. A regular cleaning schedule for incubators should be established. It is recommended that a thorough incubator cleaning occur at least quarterly. Also, standards for acceptable ranges should be established for CO_2 and O_2 levels in incubators based upon values obtained

Table 8.2 Schedule of parameters measured within the main IVF lab including embryo biopsy alcove

	Variable frequency	
Daily	**Weekly**	**Regularly**
Room temp and humidity	pH	pH new media lots
Hood surface temps	Refill dispensaries	Test UPS
Stage temps	Change water pans	Test alarms
Block heaters	Circulating water baths	Water in isolettes
Incubators	Isolettes	Clean incubators
pH		

from either a gas analyzer or preferably from obtaining actual pH values of culture media. These ranges should be based upon the laboratory's experience as well as the optimum pH level recommended by culture media manufacturers.

Protocols should be established for acceptable temperature ranges for all heated work surfaces, microscope stage warmers, tube warmers, ovens, and within all incubators [2]. It is important to check the temperatures of any surface that the culture dishes will come into contact with during Day 0–Day 6 in order to ensure that the embryos are not exposed to temperatures outside of the optimal range for embryonic development. Ranges of temperatures for each piece of laboratory equipment should be established based upon the actual temperature values obtained using a very sensitive temperature measurement device such as a thermocouple. The thermocouple probe should be placed into micro droplets for oocyte and embryo culture at various time intervals that mirror the time intervals that oocytes and embryos are kept in contact with those particular surfaces under normal conditions during embryology procedures. For benchtop incubators, a fine-wire thermocouple probe should be used to confirm temperature within micro droplets of culture dishes. This integral aspect of quality assurance helps to ensure that the embryos are never exposed to temperatures that could induce spindle defects and poor developmental rates or have other deleterious effects.

8.4 Embryo Biopsy Room or Alcove

As previously mentioned, the incidence of embryo biopsy at the blastocyst stage has increased dramatically in recent years. As a result of these changes, providing a more conducive environment for performing these procedures has become increasingly important. The reason for this is that embryo biopsy requires the use of inverted microscopy equipped with cameras and monitors situated in a quiet/non-distracting setting. A quiet setting is preferable because there needs to be constant communication between the person performing the biopsies and the person witnessing the procedures. The witness is responsible for confirming that embryos are being placed in their appropriate drops and that subsequent tissue samples are loaded in their properly labeled tubes for processing. The ability to communicate in a non-distracting environment with minimal to no distractions will likely reduce the potential for errors.

As a result of these increasing needs for specialized microscopy skills and corresponding identification of material, more laboratories are constructing either separate rooms for embryo biopsy or alcoves within the main lab to create quiet spaces to perform these delicate tasks. These rooms require the use of same building materials as found in the main embryology lab as well as clean air in a positive pressure room. The general equipment needs for these rooms usually consist of the following: laminar flow hood with a heated surface, gassing capabilities to maintain dishes outside the incubator, and a dissecting microscope. In addition, a good inverted light microscope with micromanipulators equipped with a camera and monitors is required. The need for a good anti-vibration table is of paramount importance, since the removal of tissue requires a very steady platform. Having an incubator or two for holding patient's dishes that are undergoing biopsy is ideal as well for convenience.

Tasks that should be performed on a daily basis include monitoring of temperature of the room, temperatures of headed surfaces including the hood and microscope stage, as well as gas levels for the anti-vibration table if applicable. If the lab is equipped with either benchtop or box incubators, the aforementioned QC measures should be taken on a daily basis. The laser used for embryo biopsy should be calibrated prior to use each day. Since this section of the lab contains many of the same equipment as found in the main embryology lab, the same protocols and procedures should be performed here in order to ensure that all biopsy procedures are performed in an optimum manner that does not reduce embryo viability and potential.

8.5 Media Preparation Room

The media preparation room provides a designated clean area for preparation of media solutions and weighing out chemicals used in preparing various solutions. Having a separate area for these procedures also ensures that embryos are kept away from any potentially hazardous chemicals. For those labs that do not make any media solutions, a media preparation lab provides a clean environment for preparing dishes for embryo culture, vitrification, and thawing. Some typical items that could be housed within the media preparation room include a laminar flow hood in which media or dishes can be prepared, a sterilization oven for heat sterilizing glassware, storage space for glassware, and a refrigerator/freezer used to store culture media and other items at proper temperatures. Other items commonly found in this room include a pH meter, an osmometer, and supplies. Ideally, the media preparation lab should be in a centralized portion of the lab. First aid items and an eyewash station are best located here as well. In many laboratories, media preparation rooms also house a purified water system. The media preparation room is also a good location to house all inventory records of materials used in the lab, including culture media, freezing/thawing solutions, and plasticware.

A number of tasks should be performed in the media preparation room on a daily, weekly, and regular interval basis. On a daily basis, it is recommended to check the room temperature, check the performance of the clean water system if applicable, and check the temperature of the refrigerator containing media as well as the freezer. Electronic thermometers that display the high and low range of temperatures inside the refrigerator and freezer should be used. This will ascertain that temperatures were never outside the established ranges in the refrigerator and freezer.

On a weekly basis, the eyewash station should be tested. On a regular interval basis, the water system parameters should be checked, including levels of silica and chlorine [3].

Table 8.3 Measurement of parameters within the media preparation lab

	Variable frequency	
Daily	**Weekly**	**Regularly**
Room temp	Eye wash	Media
Water system	Media inventory	Chemicals
Refrig-freezer temp	Test water system	QC sterilization oven

The water system should be cleaned according to the manufacturer's recommendations. If the lab uses a heat sterilization oven to sterilize its glassware, a contamination test should be run on a regular basis to ensure that the heat sterilization oven is working properly.

Standards that should be put into place in the media preparation room include establishment of maximum and minimum acceptable temperature ranges for the refrigerator/freezer. A diary of media preparations is recommended that should include staff that prepared and/or aliquoted the media, the date media were prepared, and expiration dates for both aliquoted and prepared media with protein. A diary or record keeping system of staff that prepared culture dishes should be kept as well. An inventory system for all chemicals used as well as a system of safe disposal of all expired chemicals should be created. A list of suggested variables that should be measured in a media preparation lab is found in Table 8.3.

8.6 Gas Room

Gas or manifold rooms, as they are commonly referred to, are usually located in a different area away from the lab. This allows the delivery of gases and liquid nitrogen by the delivery company to occur without interrupting workflow or risk of contamination of the lab. Gases and liquid nitrogen are then plumbed to the various parts of the lab.

Gas and liquid nitrogen levels should be noted and recorded on a daily basis. Based upon daily consumptions, a delivery schedule should then be put into place. On a weekly basis, delivery of liquid nitrogen and gas tanks should be confirmed as well. On regular intervals, the alarms and callback system should be checked, and the gas manifold system should be checked for gas leaks.

The standards that must be established in the gas manifold room include the minimum acceptable levels of gas and liquid nitrogen, a system to record lot numbers of gas tanks in use as well as lot numbers of tanks not yet in use and the number of tanks held in reserve at all times. Some clinics may find that a gas manifold system is safer for their laboratory as opposed to individual tanks. The manifold system provides the laboratory with the opportunity to establish settings in which the manifold system will switch the intake to a backup tank when a certain gas tank is depleted to an established minimum level. An automatic safety system serves to ensure that the incubators will always receive gas from a properly filled gas tank. In addition, standards for testing should be established to confirm that tanks will switch within the manifold system when tank pressures fall to a preset level. Today, many state regulations require that carbon dioxide levels are measured. If this is the case, then labs should have carbon dioxide detectors in their gas/manifold room and should establish testing intervals.

8.7 HVAC Room

The HVAC system is an integral component of an embryology laboratory. The HVAC system filters out the vast majority of airborne pollutants and volatile organic compounds that could enter the laboratory [3]. A well-designed clean air system is now considered an essential part of an embryology laboratory [4–6]. Most IVF centers that have been built recently use two types of filter beds in addition to HEPA filters. To physically scrub the air of volatile organic compounds, beds of carbon filters in combination with beds of potassium permanganate are often used. To remove particulate matter, a series of prefilters as well as HEPA filtration are used. On a weekly basis, pressure gradients across filter beds should be checked, and an inspection of motors and all moving parts be should be performed as well.

A number of standards must be established within the HVAC room to ensure optimum performance. A schedule for changing air filters must be established. This schedule will vary by region and depends heavily on local outside air quality. In regions with higher quality outside air, the carbon/permanganate filters can be changed approximately every 6–8 months based upon testing of the activated carbon and permanganate substrate. In areas with poor outside air quality, it is advisable to change the carbon/permanganate filters more often. It is also advisable to perform an annual check of air quality and pressurization in individual rooms within the lab. Finally, preventative maintenance should be performed on each individual component of the HVAC system based on the manufacturer's recommendation. The entire system should have preventative maintenance performed semi-annually.

8.8 Conclusion

In conclusion, daily, weekly, and regular preparations as well as establishment of standards are essential for the maintenance of ideal conditions within the embryology laboratory. Establishment of these standards should facilitate the elimination of internal laboratory factors affecting embryo quality. In other words, a rigorous assessment of internal variables allows the laboratory to focus on potential negative influences originating from outside of the laboratory. This allows the embryologist to focus on the biological variability inherent to the patients being treated at the time.

Review Questions

1. How often should surface temperatures be measured for heated surfaces in the laboratory?
2. Describe how regular measurements between benchtop incubators and big box incubators are different?
3. Why is measurement of liquid nitrogen tanks on a regular basis an important task?
4. How often should pH measurements be taken?
5. How often should chemical beds in an HVAC system be changed?

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IVF Laboratory Equipment and Culture Systems

Contents

Essential Instruments and Disposable Supplies for an IVF Laboratory

Leslie Weikert, Christa Mackenzie, and Klaus E. Wiemer

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Learning Objectives

- Understand the importance of having a well-designed laboratory space.
- Understand the importance of incubator, type, and number present within the lab.
- Review pH values and frequency of testing based upon patient volume and incubator type.
- Review the differences between workstations and isolettes.
- Review the importance of video imaging equipment in an IVF lab.
- Review the importance of uninterrupted power sources.
- Review the importance and role of all the miscellaneous equipment found within an IVF lab.
- Discuss the types, uses, precautions, and limitations associated with most plasticware found in today's lab.

Prior to selecting the type and placement of any major equipment within an IVF laboratory, the space allotted for the laboratory within the clinic must be examined thoroughly and planned with forethought. The expected patient volume, type and complexity of embryology procedures offered, and expected number of embryologists working within the space should be taken into account prior to planning the physical layout of the lab. Creating task-specific rooms within the main embryology laboratory can reduce congestion. It is important to have good workflow, allowing multiple people to work simultaneously on different procedures, safely and efficiently. For example, anticipation of traffic flow reduces congestion around incubators and microscopes. In addition, the location of air vents should also be anticipated. Airflow from air-handling systems can have a large impact on the performance of many heated pieces of equipment such as incubators, heated stages on microscopes, and heated surfaces of laminar flow hoods. The location of airflow vents is especially important in laboratories that will be using or are using benchtop incubators. If possible, a layout of equipment prior to placing air exhaust vents can help reduce potentially deleterious airflow around microscopes and incubators. The lab design should also include potential space to add additional incubators and other necessary equipment, as required.

9.1 Equipment

9.1.1 Microscopes

The following types of microscopes are essential to the operation of every IVF lab: a dissecting microscope, an upright light microscope, and an inverted microscope equipped with micromanipulators and a laser. Ideally, the dissecting microscopes are located in either a modified laminar flow hood for optimal temperature and pH control or a heated isolette with proper gas and temperature control. The dissecting microscope is used for the majority of all oocyte and embryo manipulations. With PGS and PGD becoming routine procedures performed on a daily basis in the laboratory, it is probably prudent to have multiple dissecting microscopes available. An upright microscope, which is mainly used for semen analysis and sperm preparation, can be located on a workbench outside the laminar flow hood. The inverted microscope is used for all oocyte, zygote, and embryo evaluation as well as micromanipulation procedures of oocytes and embryos. These microscopes should include differential interference contrast or modulation contrast systems and a temperature-controlled stage [1, 2]. As previously stated, an important consideration for all microscopes is the location in the laboratory relative to ventilation air ducts and air purification devices. Surface temperatures on the heated surfaces will fluctuate dramatically if cool air is allowed to vent directly onto them. It is also recommended to place micromanipulation equipment against an interior wall, well away from elevators and heavily traveled areas that may cause vibration. If possible, the use of an antivibration table for micromanipulation procedures is advised. Some laboratories perform micromanipulation procedures with their equipment on a bench top, but unexpected vibration may cause a higher degeneration rate during intracytoplasmic sperm injection (ICSI) or reduce implantation potential of embryos following biopsy.

9.1.2 Incubators

The incubator is perhaps the most important piece of equipment in the laboratory, and the selection of the style and type requires forethought as far as the initial design of the lab as a whole. Management of patient volume and workflow is crucial to optimize the recovery and stabilization of the temperature, gas concentration, and humidity [2–4]. The primary incubator still in use in most IVF laboratories is the box-type CO_2 incubator, both large and small sizes. However, benchtop incubators have started to become more popular in choice. Since it has become standard practice to culture in a reduced oxygen environment of 5% O_2, these models are available in many forms from various manufacturers. These CO_2 incubators use nitrogen gas to reduce the oxygen level. There are also multiple manufacturers offering compact benchtop incubators that use a heated chamber base plate and lid, thereby providing a more consistent thermal environment for embryo culture. The gas source is premixed and customizable by the user. The unique design of these incubators allows rapid equilibration and stabilization of temperature and pH due to the compact chamber, unlike the large upright chambers of traditional water- and air-jacketed incubators. Recently, time-lapse incubators (Embryoscope®, Esco Miri®, Geri®) are gaining in popularity despite their high price tag. There have been a few publications that have suggested that by monitoring embryo morphokinetics, it may be possible to select embryos that may be less likely to carry chromosomal abnormalities, thus improving clinical outcomes [5].

If designing a new lab, it is ideal to select the type of incubators that will be used in the lab early in the design process in order to place the appropriate gas lines in the proper location for present and future use. Depending on the culture system, a combination of incubator styles may be a practical choice. It is important when designing a new lab to be sure to include gas outlets for future incubators as well.

A gas analyzer is used to monitor the actual gas content within an incubator, and these measurements should be taken as part of the daily quality control program. The measured value is compared to the digital value displayed for each individual gas, and adjustments are made as required. A pH meter is used in conjunction with the gas analyzer to determine the appropriate settings for each incubator. The commercially prepared media that are available from most manufacturers generally measure at a pH of 7.2–7.4 post-equilibration. Each incubator should be checked on a regular basis with a control lot of medium to confirm that the pH of medium cultured within that incubator measures within the laboratory's established acceptable range. It is suggested that each new lot of bicarbonate-buffered medium received be pH checked prior to use. These pH values should be the main determinant of the CO_2 set point of each incubator. pH values should be taken as well when the water humidification systems in benchtop incubators are replaced to ensure the systems are functioning correctly.

9.1.3 Workstations

It is recommended that all IVF procedures are performed within a controlled environment. One method used to accomplish this is to use modified laminar flow hoods. These workstations provide protection against particulate and microbiological contamination while also providing a temperature-regulated surface for embryo/oocyte manipulation. Typically, these hoods are fitted with a heated surface that uses either a heated circulating water bath or electrical circuitry. They also are fitted with gassing and humidification devices that enable the embryologist to maintain the pH of culture media while dishes are outside of the incubator. An additional benefit of these systems is they allow the embryologist to sit at the workstation and work in an unencumbered environment. However, these hoods require calibration of the heated surfaces and a source of gas for the gassing devices.

Some laboratories choose to use a modified isolette that provides temperature and pH regulation within a closed system. These isolettes are modified in such a manner to allow a dissecting microscope to be placed within the isolette. The advantage of the systems is that the chamber is completely enclosed so there is less chance of pH drift and temperature fluctuation. These systems also allow embryologists to work without the use of HEPES-buffered media since the chambers are typically flooded with 5–7% CO_2 gas. Isolettes are also available in portable models and are useful when the procedure room is not adjacent to the laboratory. The main disadvantage of these systems is that they are cumbersome to use, can be quite restrictive as far as hand movements, and require the embryologist to stand throughout the tasks at hand. The heated surfaces should be checked as well, as temperature fluctuations have been noticed in these isolettes.

9.1.4 Video Equipment

Due to the nature of this field, it is advisable to have video monitoring equipment at each IVF workstation. In this way, a witness can watch procedures as required and confirm patient identity as necessary. This establishes a visual chain of custody for such procedures as insemination of oocytes, change of oocytes/embryos to new dishes, and embryo biopsy. It is recommended that visual conformation of all steps associated with embryo biopsy and the processing of the cells be performed.

If this equipment is also included in the patient procedure room, the patient can be included in the identification procedure for retrievals and transfers. It is also customary to provide each patient with an embryo photo at the time of embryo replacement. A camera and/or digital video recorder (DVR) may also be used to record other procedures in the laboratory.

9.1.5 Cryopreservation and Storage

Cryopreservation technology in human IVF and the trend toward extended culture have changed the emphasis from cryopreservation of early-stage embryos using controlled-rate freezing to vitrification of all stages of oocytes and embryos. The equipment needed for most vitrification protocols is minimal but varies depending on the protocol used. In order to ensure clear and legible writing on cryostraws and devices, a label maker should be used. Marking pens eventually wear or rub off, jeopardizing the correct identification of patient specimens. This is a huge risk, and the cost of a labeling machine is less compared to the cost involved if a sample is misidentified! Other commonly used supplies for cryopreservation include a heat sealer for cryostraws, plastic dewar for liquid nitrogen, protective gloves, and a thermocouple thermometer. It is recommended that a small warming oven be placed in close proximity of the dissecting microscope used for thawing of vitrified samples to reduce the possibility of the thaw solutions cooling prematurely.

There are many variables to take into consideration when choosing cryostorage tanks including patient volume and whether the clinic plans to offer short- or long-term storage for their patients. If long-term storage is planned, an investment should be made in large tanks with autofill capability. For smaller programs, individual tanks on roller bases can be used but should be monitored individually for temperature or nitrogen level by alarms. It is also a good idea to have a vapor tank on hand to transport specimens. The vapor tank can also be used for certain types of embryo and oocyte vitrification procedures. All equipment housing gametes or

embryos should be monitored 24/7 by an alarm system, which automatically calls out in the event of an equipment malfunction.

9.1.6 Power Related

An uninterrupted power source (UPS) is traditionally used as a short-term power supply in the event of a power failure. They are most beneficial in protecting sensitive electronic equipment from power fluctuations, brownouts, and line noise. A well-planned laboratory will have all incubators and tissue-freezing equipment running through UPS power. A UPS is very effective at "cleaning up" electricity and, in doing so, extends the lifespan of electronics. IVF laboratories should have backup electrical generators that provide electricity in the event of a power outage. UPS batteries should be checked annually and replaced accordingly. Generator output should be determined after the total amount of equipment that needs to be functional when power outages have been established. Whenever possible, equipment needs should be determined during the construction phase of the lab so that the equipment can be wired and placed on emergency breakers. This way, when the generators turn on, the essential equipment will be on emergency power. In addition, the UPS systems will hold the incubators and other crucial equipment until emergency power is available. The generator should be tested on a monthly basis, and at least once a year, the lab should be run exclusively on emergency power.

9.1.7 Miscellaneous Equipment

It is generally accepted today that contaminant-free air is an integral part of an IVF laboratory in order to consistently produce high-quality embryos. Therefore, an HVAC system is an essential part of today's IVF center. HVAC systems used in IVF laboratories remove volatile organic compounds (VOCs) and chemical air contaminants (CACs) as well as particulate matter. If an HVAC system is not an option, mobile air purification devices (including carbon and HEPA filters) are available. Some of CO_2 units available today remove VOCs, CACs, odors, mold, and particulates.

There are three common pieces of equipment used for oocyte retrieval in most IVF clinics: a dry bath incubator, an aspiration pump, and a warming oven. Dry bath incubators provide uniform dry heating for test tubes. Interchangeable modular heating blocks provide the flexibility of accommodating various test tube sizes and quantities. A dry bath is useful for many purposes, and it is practical to have multiple dry baths in different areas of the lab. They can be used to warm media and oil used for micromanipulation procedures, as well as test tubes used during oocyte retrievals. A forced-air incubator with microprocessor control is ideal to warm media, test tubes, and modular warming blocks used during oocyte retrievals and embryo transfers. An aspiration pump with foot control is used with either a single- or double-lumen needle to puncture follicles and collect oocytes during the oocyte retrieval. The pump should ideally provide smooth, low-volume suction at a predetermined negative pressure. This pump may also be used in other procedures such as endometrial sampling or cyst aspiration.

A high-speed multipurpose centrifuge is used during semen processing for IVF, IUI, or diagnostic procedures. A high-end centrifuge with an internal microprocessor simplifies operation, ensures repeatable results for continued success, and alerts operators when periodic maintenance is due. Do not place the centrifuge in an area where vibration would cause problems for other pieces of equipment.

Oocytes and early embryos (particularly in the pre-compacted stage) are extremely sensitive to their environment and fluctuations in temperature (among other factors) may alter their cellular physiology and compromise viability [2]. Therefore, a thermocouple thermometer is essential for checking surface temperatures of microscope stages and heated work surfaces. This instrument is important for initial calibration and daily quality control (QC). It is also handy for checking temperatures of media that are warmed to certain temperatures prior to use and the temperature in microdrops of culture media under oil when calibrating equipment or performing QC procedures. A mercury or spirit-filled thermometer is necessary to monitor the temperature of each incubator, dry bath, warming oven, water bath, refrigerator, and freezer. Digital thermometers are also available and can be used for some of these items. A certified thermometer is required for annual calibration of all thermometers that are not sent to outside facilities for calibration.

The size and type of refrigerator and freezer needed should be based upon patient volume, kind of materials stored, and type of tests/procedures offered. However, refrigerators that can keep culture media and other important materials within their ranges of temperature for storage should be selected. The storage temperature range recommended for most commercial media is 2–8 °C. In many instances, a refrigerator for only culture media and related products is warranted. The type of freezer selected should be based on type of materials to be stored, sensitivity to temperature changes, and anticipated length of storage. Given the increasing popularity of PGD and PGS and the need to store particularly sensitive tissue specimens, a specialized lab freezer may be a practical investment.

The following pieces of equipment may not be necessary but can be useful. A water purification system is convenient as a supplier of unlimited ultrapure water for incubator humidification pans. It is a significant investment and may not be necessary for most IVF programs. An osmometer is used to measure the osmolality of any given solution. The osmolality of commercially prepared media generally falls between 260 and 290. Osmolality is not a commonly performed test in most labs; however, some of the proficiency testing services include this as a test for culture media. An analytical balance might be useful to weigh out chemicals used for making media for various procedures.

9.2 Disposable Supplies

9.2.1 Plasticware

Disposable plasticware (Petri dishes, culture/centrifuge tubes, flasks, serological pipettes) is utilized extensively throughout the IVF laboratory for everything from oocyte/embryo culture to storage of various solutions. Too often, when designing a new laboratory, inadequate space is allotted for storage of plasticware and other disposable supplies. It is beneficial to have a sizeable storage room adjacent to the lab. When supplies are received, they should be removed from any brown cardboard packaging. Brown cardboard is frowned upon in most any hospital/medical storage areas due to its "dirty" connotation. Ideally, the individual packages of plasticware are slightly opened to begin the off-gassing process in the storage area. The amount of each item to stock is a function of the budget, the number of patients cycling, and the amount of storage space available. If possible, it is practical to purchase multiple boxes of the same lot number as it will save time and money as far as the QC required for each new item.

Each lab should develop an inventory database in order to ensure an adequate supply of all necessary materials are on hand and to create a rotational system where older stock is used first. As new lots are received, they are logged into the database with the date of receipt, specific lot number, product expiration date (if applicable), and testing status. As each new lot is put into use, the start date is also logged into the database. Used in this manner, the database may be a useful tool if a negative trend in the lab is noticed, such as poor embryo development.

There are many products manufactured specifically for IVF that are marketed as "tested," meaning that they have passed some sort of bioassay. However, the assays themselves and their criteria for passing should be reviewed. They may not meet the guidelines established in a particular laboratory as acceptable for use. In addition, these same "pre-tested" materials may become toxic if stored or shipped in suboptimal conditions [3].

Most companies and IVF laboratories use either the mouse embryo assay or the 24-h sperm survival assay (or a combination of the two) to test media and lab disposables. While the two-cell mouse assay used to be the standard, it has become evident that the one-cell assay is much more sensitive in detecting a problem [6, 7]. The strain of mouse used can also impact the test sensitivity. Other factors that can affect the test include the presence of amino acids and the concentration of protein in the culture medium. Both are able to chelate toxins and possibly ameliorate their effect [8].

An important aspect concerning plasticware is that it should be removed from its packaging to allow for the off-gassing of VOCs for at least 24 h and preferably longer, prior to use. VOCs such as styrene and toluene are present in Petri dishes [9] for a period of time after removing from packaging. These compounds are highly soluble in oil and can affect embryo development. The levels in any incubator are directly proportional to the number of dishes currently housed in the incubator [10]. In the case of certain Petri dishes, tubes, and flasks, there also may be residues from ethylene oxides used in the sterilization process [11]. In addition to off-gassing, rinsing of dishes or material used to store products such as media or protein for embryo culture is recommended to remove debris and contaminants (mostly from the manufacturing process). Disposable syringes used for embryo transfer may benefit from being "leached" in culture medium for a short time prior to use.

Plasticware for embryo culture is available from many large manufacturers (BD Falcon™, Nunc™, and Corning™) and more recently from some specialty companies (LifeGlobal and Vitrolife) and is a matter of personal choice based on the type of culture system the laboratory director elects to use. The majority of oocyte and embryo culture systems currently in use are oil and microdroplet systems. Many types of dishes or multiwell plates can be used for this type of culture system. Plasticware can be classified into categories based upon function and purpose. For example, plasticware used for oocyte identification tends to have a larger surface area in order to expedite the identification of the cumulus-oocyte masses. These dishes range in size from 60 to 100 mm in diameter.

Dishes for oocyte culture tend to be approximately 60 mm in size. This size dish allows for larger drops to accommodate the cumulus-oocyte masses prior to and after insemination using either conventional means or ICSI. A 60 mm dish can easily accommodate most, if not all, of a patient's oocytes. The main issue to consider when selecting a dish manufacturer for microdroplet culture is how the droplet shape will present itself. Many times, the drops will flatten out with time. This is mainly determined by the presence or absence of an electrical charge on the dish. The type of oil used also may have an effect on droplet integrity.

Dishes for embryo culture tend to be smaller and range in size from 35 to 60 mm. Multiwell and microdrop dishes are used for embryo culture as well and there are many new designs available.

9.2.2 Oocyte Retrieval Needles

The type (single or double lumen) and size (16 g or 17 g) of oocyte retrieval needles in use today are a matter of choice and that decision will probably be made after consulting with the physician. All work very well, and the incidence of damage to oocytes caused by needles is remote when properly used. The pressure used to evacuate the follicles is critical, and damage to the cumulus-oocyte complex is more likely to occur with increased pressure.

9.2.3 Miscellaneous Disposables

One of the most important decisions to be made as far as disposables is what tool will be used for oocyte and embryo manipulations. Pulled glass pipettes were very popular for a

long time. If the physician is able to sanitize and sterilize them on-site, they are an extremely cost-effective tool; however, their preparation is labor intensive and has its own cost as far as the ultrapure water required for preparation. They can be purchased pre-prepped and sterilized. Their one drawback is that there is a rather steep learning curve as far as pulling the size desired for the procedure the physician is performing.

There are many other tools offered on the market today (Stripper® Tips, Flexipets®, EZ-Tips®), but they also bear a large price tag. The use of a mix of materials may be a wise choice. Other materials required for a lab to run efficiently are items such as catheters for embryo replacement, micropipets for ICSI and embryo biopsy, serological pipettes, filters for solutions, and supplies for cryopreservation such as canes and goblets. The choice of which of these materials are used is very dependent on how the lab director manages the lab, the caseload, the number of staff available to perform the procedures, as well as budget.

9

9.3 Conclusion

This chapter is an overview of the essential materials required to set up a clinical human IVF laboratory. If a new lab is being built, it very important to select the major pieces of equipment at the outset of the design phase so that the floor plan can accommodate them in an efficient and well-thought-out manner. This will also help with designing casework as well as budgetary requirements. The type of equipment selected should be a process that involves both the physician and the laboratory director so that the center can maximize usage of the equipment and space.

The selection of disposables is almost an arbitrary selection that should be based upon the needs of the director and staff. An efficient inventory system and QC program will ensure that proper quantity and quality of materials are on hand at all times.

Review Questions

1. What impact can airflow have on heated surfaces?
2. What is the most important piece of equipment in an IVF lab?
3. How often should pH measurements be taken?
4. What are some of the advantages and disadvantages of workstations as compared to isolettes?
5. Why is it important to have video equipment in today's IVF lab?
6. What role(s) do uninterrupted power sources serve in an IVF lab?
7. How should plasticware in the lab be inventoried and managed in the lab?

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CO_2 and Low-O_2 Incubators

Marius Meintjes

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Learning Objectives

- To review and to compare the physical characteristics of traditional and second- and third-generation incubators for the IVF laboratory
- To assess benefits of low-O_2 concentration incubators relative to "traditional" CO_2 incubators
- To evaluate the need for different embryology laboratories for certain types of incubators to best serve their functions
- To review how to perform maintenance on the incubators and what quality control measurements are important

Embryo incubators can be considered the heart of any in vitro fertilization (IVF) laboratory. Understanding the strengths and weaknesses of our incubators is invaluable as a tool and essential to optimize clinical IVF outcomes. Whether we consider using our existing incubators to their full potential or trying to decide if we have ample incubators for our current needs, it is imperative to have current knowledge of what is available in the marketplace and to make some sense from the myriad of specifications and claims on the various product brochures. When we are fortunate to consider the purchase of a new incubator or, even more so, when constructing a new IVF laboratory facility, it is critical to review potential costly infrastructure demands, depending on the type of incubator under consideration. The historical standard is the conventional thermoconductivity (TC) sensor, water-jacketed CO_2-only incubators. With some level of blastocyst culture becoming an industry standard and more publications in favor of reduced-O_2 culture, reduced-O_2 incubators with their requirements for premix or N_2 infrastructure should be considered. With second- and third-generation top-load benchtop incubators now available from multiple vendors, we are challenged with a paradigm shift away from larger conventional incubators. Should we choose to go this route, the laboratory design must be able to accommodate altered floor space configurations, additional bench space, dedicated premix or N_2 gas supply lines, and, in some cases, supply gas pressures higher than those we are accustomed to. Knowing that more than one sound approach to IVF can yield excellent results, the goal is not to recommend specific products or solutions but rather to suggest principles and general guidelines useful to everyday decision-making.

10.1 Do We Need Low O_2?

The embryos of various mammalian species, including the human, are not exposed to O_2 concentrations in excess of 8% in vivo [1–3]. Not unexpectedly, numerous studies in animals have consistently demonstrated that embryo cultures in low-O_2 concentrations markedly improve in vitro embryo development and subsequent pregnancy outcomes when compared with similar cultures in atmospheric O_2 concentrations [4–8]. However, when culturing human embryos in lower-O_2 concentrations, the expected improvement in measured laboratory parameters [9, 10] or clinical outcomes [11] is frequently not observed. The human studies that found no benefit in early clinical outcomes with a lowered-O_2 tension in the incubators were mostly associated with embryo transfers on day 2 or on day 3 [10]. When transferring blastocysts and, specifically, when observing live births, a clear beneficial effect of lowered-O_2 embryo culture is consistently observed [12] with an increase in live-birth rate of almost 15% [13]. Stated in another way, the embryos of only seven patients have to be cultured in a reduced-O_2 culture environment to result in one additional live birth.

Numerous animal studies have found that even a brief exposure of pronuclear or cleavage-stage embryos to atmospheric O_2 can be detrimental even though it may only be manifested at the morula or blastocyst stage [14, 15]. It becomes apparent that even embryos transferred on day 2–3 might benefit from culture in reduced O_2. Selective later-stage reduced-O_2 culture may be an ill-advised approach, since the damaging effects of atmospheric O_2 may already be present in early embryos but only manifested at later stages of development and expressed as altered metabolism or gene expression [16] with reduced viability. Furthermore, low-O_2 culture appears to improve the frequently observed skewed live-birth sex ratio (more boys than girls) when culturing blastocysts [17]. Available live-birth data clearly suggest that human embryos should be cultured in a low-O_2 environment through all in vitro stages of development to ensure the highest live-birth rate and the most appropriate gene expression. Using modular chambers inside existing conventional CO_2-only incubators provides one way to culture embryos in a low-O_2 environment. With the clear clinical indications for low-O_2 culture, only incubators capable of reducing the O_2 concentration will be reviewed in this chapter.

10.2 Benchtop (Top-Load) Incubators

It was reported that the implantation rate was increased from 10% to 14% and the pregnancy rate from 19% to 32% when culturing human embryos in MINC (Cook IVF) incubators in an atmosphere of 6% CO_2, 5% O_2, and 89% N_2 instead of in standard water-jacketed incubators in an atmosphere of 5% CO_2 in air [18]. It is likely that the beneficial effect seen in this study was due to the faster recovery rate demonstrated by the MINC incubators rather than by the lowered O_2 concentration or the higher CO_2 concentration. The benchtop incubator design allows for the direct transfer of heat through contact surfaces to the embryo culture dish unlike the indirect convective transfer of heat through surrounding air in a conventional incubator [19]. This helps to explain why the temperature recovered within 5 min in an MINC incubator compared with roughly 30 min for a standard, water-jacketed incubator after a single door opening [20]. With premixed gases immediately purging the microchambers of the MINC and Planer BT37 incubators, or the constant recirculation of custom-mixed gases through the microchambers of the

Table 10.1 A comparison of some features of common top-load benchtop incubators

Benchtop incubator	Gas supply	Humidity control	List price	Capacity (patient units)	Digital recording	Alarms
Cook MINC	Premixed	Yes	$13,500	2 × 4	+	+
Planer BT37	Premixed	Yes	$11,900	2 × 4	+	+
FIV-6	Premixed	Yes	$4600	1 × 2, 1 × 4	–	–
K-Systems G-185	Built-in gas mixer	No	$32,000	10 × 2	+	+

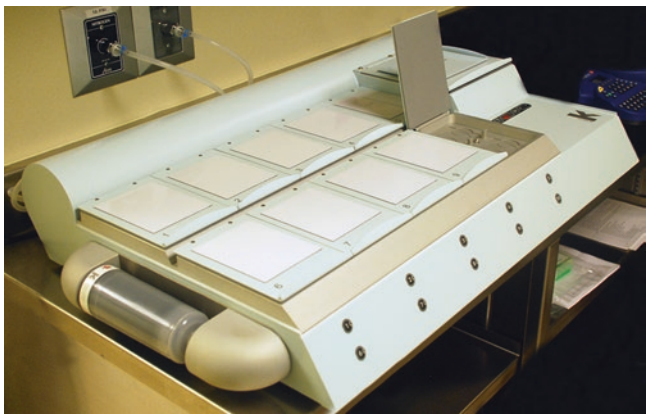

Fig. 10.1 An example of a benchtop, top-load incubator, allowing for direct heat transfer to culture dishes from all sides, fast gas-phase recovery, and in-line N_2 and CO_2 filtration

K-Systems G-185 (Table 10.1) incubator, it is understandable that the targeted O_2 and CO_2 concentrations are reached much faster in benchtop incubators. The smaller footprint size of benchtop incubators and patient-specific chambers allows for use in confined spaces with minimal disturbance of the culture environment when accessing multiple culture dishes (Fig. 10.1). The minute amounts of gas needed to stabilize a benchtop microchamber and the relative lack of moving parts result in significant savings in maintenance and gas supply, estimated to be in excess of $1000 per incubator per year. With a single gas source now lasting longer, the gas supply becomes a lesser source of variation over time. Benchtop incubators are very easy to clean and maintain and are less prone to fungal and bacterial contamination compared with bulky conventional incubators with multiple moving parts, exposed sensors, and abundant internal insulation material.

Benchtop incubators do pose significant quality control and quality management challenges, unique to their size and design. In the event of a sustained power failure or unexpected loss of gas supply, limited opportunities exist to redeem patient material, even with a timely alarm signal. Unlike conventional water-jacketed incubators that gradually lose temperature (~1 °C/h) and gas content over time, the destabilizing effect of an untoward event in a microchamber of a benchtop incubator is immediate. The BT37 benchtop incubator has a standard 2-h battery backup capability, reducing some risk in the event of a power failure. Periodic temperature, gas concentration, and pH checks are more difficult in benchtop incubators due to the small chamber size and relative inaccessibility of the microchambers. The temperature can be verified without much trouble by feeding an appropriate K-type wire thermocouple into the chamber. The Planer BT37 is equipped with specific thermocouple measuring ports in the lid and the chamber to assist with temperature verification efforts. Gas supply quality control is less relevant in models using a premix gas (MINC and Planer BT37). Once the purity and accuracy of the source gas has been verified, no need should exist to continuously test the gas supply. However, periodic CO_2 and O_2 concentration measurements are essential for the K-Systems G-185 to verify the consistency of the built-in gas mixer. The G-185 is equipped with a customized sampling lid and sample ports, which allow for convenient measurement of CO_2 and O_2 gas concentrations. Conventional CO_2 and O_2 fyrite solutions are not usable in benchtop incubators, since the sample size required for a measurement is larger than the capacity of the incubation chamber. Capnometers and in-line continuous-read CO_2 and O_2 sensors have been proposed as more expensive alternatives. pH measurements of culture media in benchtop incubators are more challenging. The pH can be measured in situ with a micro-pH probe through the sampling lid of the G-185, or, alternatively, an immediate on-site blood-gas analysis and pH reading can be obtained.

The MINC, FIV6, and Planer BT37 incubators use premixed gas only, which makes it difficult to incorporate these incubators into existing laboratories without previously installed premix gas lines. Options are to bring dirty premixed gas cylinders into the laboratory or to install an additional premix gas delivery manifold and pipeline. The recommended supply pressure of premixed gas is ~22 psi, which is significantly higher than the ~10 psi used for conventional incubators. The K-Systems G-185, with its own gas mixer, can be incorporated more easily into existing laboratories with standard-pressure (10 psi) CO_2 and N_2 supply lines. When using premixed gas, the CO_2 concentration can be adjusted for specific batches of media or different stages of embryo culture only by replacing the supply tank with a new one. The G-185 allows instant customization of the CO_2 concentration by simply adjusting the CO_2 set point. The MINC and BT37 pass the premixed gas through a water reservoir to provide chamber humidification. The G-185 does not have a similar feature and humidification, and if desired, it can only be obtained with an open

water-filled petri dish in each chamber. The lack of humidity is not important for media kept under oil since there is no detectable change in osmolarity for at least 48 h. Medium changes every 48 h render humidity control effectively irrelevant in the G-185. However, culturing or warming media in the G-185 without an oil overlay should be avoided. The FIV6 is representative of the first generation of benchtop incubators with less accurate temperature control and higher premix gas flow rates and, therefore, should not be used for prolonged embryo culture. All premixed gas delivered to the MINC or BT37 should be of the highest quality with HEPA- and broad-spectrum gas filtration. The K-Systems G-185 recirculates the chamber atmosphere to conserve gas but increases the risk for VOC build up on the inside. The G-185 incorporates a HEPA and activated carbon filter, but filtration of alcohols, ketones, aldehydes, and other common polluting gases is compromised by the lack of permanganate in these filters.

10.3 Water-Jacketed and Direct-Heat (Air-Jacketed) Incubators

The ability of a water-jacketed incubator to preserve chamber temperature after a power failure is superior, allowing more time to rescue embryos. Thermo Scientific Inc. reported, that during a power failure simulation of the Forma Series II water-jacketed incubators in an 18 °C ambient environment, the drop of the chamber temperature was only 1 °C per hour (from 37 to 36 °C) and approximately 7.6 °C in 10 h. Under the same conditions, a similar air-jacketed incubator lost 3 °C during the first hour (from 37 to 34 °C) and 17 °C in 10 h. Decontamination cycles are only possible with direct-heat, air-jacketed incubators. Water-jacketed incubators are unable to be heated to the levels (> 90 °C) necessary to achieve chamber sterilization. Water-jacketed incubators are heavy and large, gas-phase recovery may be compromised, and maintenance of the water jacket can be challenging. The typical elaborate chamber insulation in these incubators can become moist over time and serve as a continuous source of fungal contamination.

10.4 Size of Incubator

As a rule, the smaller the incubator, the faster the gas-phase recovery with the fastest recovery to be expected from the top-load, benchtop incubators such as the MINC and K-Systems G-185. This is not always true, as a larger incubator with an infrared (IR) CO_2 sensor (Fig. 10.2) can have a faster CO_2 recovery time than a smaller incubator with a thermoconductivity CO_2 sensor, e.g., the 170 L Sanyo 18 M has a faster recovery time than the 49 L Sanyo 5 M (Table 10.2). Smaller air-jacketed incubators have a smaller footprint and, due to their light weight, can be stacked and incorporated into integrated workstations. Most of the larger incubators (>150 L) were originally designed for bulk cell-

Fig. 10.2 A 170 L infrared CO_2 sensor, conventional air-jacketed incubator. Faster CO_2 recovery can be expected with a large infrared sensor-equipped incubator than with a smaller incubator with a thermoconductivity CO_2 sensor

culture and microbiology applications with limited door openings anticipated per day. When culturing human embryos, these same conditions are rarely observed with multiple door openings per day common and a strong incentive to limit the number of patients per incubator. In human IVF applications, the number of patients per incubator should be limited to reduce risk in the case of incubator malfunction, to decrease the likelihood of sample confusion, and to maintain the most optimum culture conditions by reducing the number of door openings per day [21]. The number of cases to be cultured in a single incubator should be determined not only by the mere size of the incubator but also by the recovery time for the specific incubator as a function of the size of the chamber, the type of CO_2 sensor, the method of humidification, and the clinic-specific culture protocols. As an example, a large 184 L Forma incubator with a thermoconductivity CO_2 sensor and passive humidification will have trouble maintaining stable culture conditions with more than 3–4 sets of embryos at any given time. Keeping this in mind, much of the space of a large, conventional, water-jacketed incubator is unusable.

Table 10.2 A comparison of characteristics of common, conventional low-O_2 incubators

Model	CO_2 sensor	O_2 sensor	Humidity control	Jacket	Size (L)	Decon cycle	HEPA filtration	UV	Copper
Astec APM-30DR	IR	Fuel cell	Passive	Water	32	–	Chamber	–	–
Astec APM-50DR	IR	Fuel cell	Passive	Water	50	–	Chamber	–	–
Binder CB 150[a]	IR	Zirconium	Passive	Air	150	180° overnight	–	–	–
Binder CB 210[a]	IR	Zirconium	Passive	Air	210	180° overnight	–	–	–
Forma 3130	TC	Fuel cell	Passive	Water	184	–	Chamber	–	–
Forma 3140	IR	Fuel cell	Passive	Water	184	–	Chamber	–	–
Galaxy 170R[a, b]	IR	–	Active	Air	170	120° 4 h	CO_2	+	100% cu
Galaxy 48R[a, b]	IR	–	Passive	Air	48	120° 4 h	CO_2	–	–
Galaxy 14S[a, b]	IR	–	Passive	Air	14	–	CO_2	–	–
Heracell 150i[b–d]	IR	Zirconium	Passive	Air	150	90° 9 h	–	–	100% cu
Heracell 240i[b–d]	IR	Zirconium	Passive	Air	240	90° 9 h	–	–	100% cu
Heraeus BBD 6220	TC	Zirconium	Active	Air	220	180° 3 h	–	–	–
NAPCO 8000	IR	Fuel cell	Passive	Water	184	–	Chamber	–	–
NuAire 4950	IR	Fuel cell	Active	Water	188	–	Chamber	–	–
Sanyo MCO 18 M	IR	Zirconium	Passive	Air	170	–	–	+	Cu-enriched stainless steel
Sanyo MCO 5 M	TC	Zirconium	–	Air	49	–	–	+	Cu-enriched stainless steel
Thermo Scientific 8000	IR	Fuel cell	Passive	Water	184	–	Chamber	–	–
Thermo Scientific 8000 DH	IR	Fuel cell	Passive	Air	184	140° 2 h	Chamber	–	–

[a]Fanless
[b]Data logging
[c]Panless water reservoir
[d]Touch screen

10.5 Carbon Dioxide Measurement and Control

Two main CO_2 sensor types are commonly found in modern-day incubators: thermoconductivity (TC) and infrared (IR) sensors. TC sensor-equipped incubators are less expensive; however, CO_2 recovery is dependent on the chamber's relative humidity (RH). When opening and closing an incubator set at 6% CO_2 and equipped with a TC sensor, the digital display will show a recovery of the CO_2 concentration within 5 min. However, when measuring the CO_2 concentration with an independent device, the actual CO_2 concentration will be closer to 2% and only approach the 6% original setting once the RH move toward 90%. Depending on the chamber size and humidification system, it may take 1 h or more to reach a 90% RH. When culturing embryos under oil, the RH level per se is less important, except that it directly affects the CO_2 recovery time in a TC-equipped incubator. Some manufacturers, such as NuAire, Galaxy, and Heraeus, improve the CO_2 recovery time for their TC sensor models with active-spray humidification systems to significantly reduce the humidity recovery time. Interestingly, the smaller 49 L Sanyo MCO 5 M incubator, only available with a TC sensor, has a longer CO_2 recovery time than the larger 170 L, IR sensor-equipped MCO 18 M (Table 2). Sanyo improved the CO_2 recovery time of the MCO 5 M model significantly over that of conventional TC sensor incubators by housing the TC CO_2 sensor in a smaller side chamber. The RH recovers much faster in the small side chamber than in the main MCO 5 M incubation chamber with the CO_2 recovery time now reduced to less than 15 min. The CO_2 recovery of IR sensor-equipped incubators is humidity independent and takes only minutes. Therefore, a larger IR sensor-equipped incubator can be used for more patients at a time compared with the conventional TC sensor-equipped incubator while maintaining stable culture conditions. With a much faster

CO_2 recovery, even after multiple door openings, IR sensor-equipped incubators are the incubators of choice for today's IVF applications.

10.6 Oxygen Measurement and Control

There are several different ways of measuring O_2 using technologies such as zirconium dioxide ceramic, electrochemical (also known as electrogalvanic fuel cell), infrared, ultrasonic, and, more recently, laser technology. The two commonly used methods in today's IVF incubator applications are zirconium dioxide (ZrO_2) sensors and electrogalvanic fuel cells. The zirconium sensor is also referred to as the "high-temperature" electrochemical sensor, using a solid-state electrolyte (ZrO_2) to generate a current directly related to the O_2 concentration. Zirconium sensors do have a faster response time compared with fuel-cell sensors and, typically, are more expensive. Due to the high temperatures of operation, the life of the sensor can be shortened by on/off operation causing sensor fatigue. The lifetime of a zirconium sensor, generally, is much longer than that of the fuel-cell sensor.

The fuel-cell sensor is an ambient temperature sensor and functions very much like a battery, making use of a chemical reaction between KOH and O_2 to create a current directly proportional to the O_2 concentration. Unlike the solid-state zirconium sensor, a fuel-cell sensor typically presents as a small, partially sealed, cylinder that contains two dissimilar electrodes immersed in an aqueous electrolyte such as KOH. Electrogalvanic fuel cells have a limited lifetime, similar to a battery, which is reduced by exposure to high concentrations of O_2 (higher rate of chemical reaction consuming the fuel cell). The lifetime of the sensor can be increased by leaving it in open circuit when not in use, but unlike the zirconium sensor, it takes about 20 min to become functional after reconnection. With the advance in mechanical designs, refinements in the electrode materials, and enhanced electrolyte formulations, the life of the fuel-cell sensor is significantly extended over earlier versions and similar to the zirconium sensor is recognized for its accuracy over a wide range of O_2 ranges. Both zirconium and fuel-cell O_2 sensors are well suited for IVF applications.

10.7 Quality Control

10.7.1 Temperature

Incubator temperature drives the metabolic rate of the embryos in culture. Some authors believe that the incubation temperature should be kept safely below 37 °C at 36.7 °C [22]. Another way of thinking is to set the incubator at 37.1 °C to prevent the temperature from dropping below 37 °C for prolonged periods when opening and closing the incubator door. Regardless of the target temperature, fluctuations in temperature should be avoided. As discussed above, the direct transfer of heat from below and from above in top-load incubators allows for a much faster recovery of heat compared with conventional incubators [19, 20]. Heat stability in conventional incubator chambers can be improved with the use of passive heat reservoirs such as the Incuplate (Cook). It has been demonstrated that the temperature recovery time for conventional incubators with Incuplate devices approaches that of top-load incubators and significantly outperforms conventional incubators without Incuplate [19]. The volume of the culture medium, the volume of the oil overlay, and the use of a culture dish lid significantly affect heat retention and heat recovery time, regardless of the incubator type. In general, heat is conserved when using a lid and when culturing in larger volumes of medium and oil [23]. The tolerance range for temperature variation in the stabilized incubator chamber should be very small (±0.1 °C). The chamber temperature should be monitored daily with an independent device, preferably with a certified thermometer. Perforated shelves are essential to prevent a temperature gradient within the incubator, especially for brands without fan-mediated air circulation such as Galaxy (Table 10.2). Even with perforated shelves, the temperature may be different on different shelves. Therefore, the incubator chamber temperature should be calibrated and verified specifically in the same location destined for embryo cultures.

10.7.2 pH

Incubators should not be set for a specific CO_2 concentration but rather with the goal to achieve a target pH such as that recommended by the media company for a specific medium. The pH of the culture medium is not necessarily a function of the percentage of CO_2 in the incubator chamber but rather of the partial pressure of the CO_2 in the embryo culture environment. The partial pressure of CO_2 is affected by the height above sea level, and therefore, the set CO_2 concentration required to achieve the target pH in a batch of medium may be different for laboratories in different sites. Since different batches of medium may differ slightly in buffering capacity, the actual pH at a specific CO_2 setting should be verified for each new batch of medium. In addition, consistency in pH values at a given setting between incubators should be confirmed periodically. As a guideline, the target pH should never exceed 7.4, even when moving or evaluating embryos outside the incubator. The repeatability and dependability of pH measurements are critical and, sometimes, challenging. The pH probe should always be calibrated with buffers that bracket the target pH, and importantly, calibration should take place at the target temperature (37 °C). In situ pH measurements of the culture medium, as used for IVF inside the incubator after overnight equilibration, consistently yield reliable results. An instant pH measurement with a calibrated blood-gas analyzer of appropriately sampled, equilibrated culture medium is a dependable alternative. A blood-gas analyzer has the added benefit of also measuring the partial pressure of O_2 (PO_2) in the culture medium.

10.7.3 Carbon Dioxide

CO_2 is usually delivered in the liquid form in H-size, medical gas cylinders. The CO_2 then evaporates from the pressurized CO_2 liquid, yielding a very clean supply of CO_2 for use in IVF incubators. All particulate and gaseous impurities mostly stay behind in the liquid phase. When the cylinder runs empty (no more liquid), these impurities are released at once with the potential to saturate and bypass any in-line gas-phase filters. It is, therefore, good practice to prevent CO_2 supply cylinders from running completely empty before switching to the reserve. Because of the liquid state of the CO_2 supply and a constant pressure in the air space of the tank, one will not see a notable drop in manifold pressure until shortly before the cylinder runs empty (Fig. 10.3). To prevent the CO_2 cylinder from running empty and to ensure a timely switch to the reserve cylinder, one must set the changeover pressure on the manifold above 50% (~500 psi) of the original tank pressure (~850 psi). Alternatively, the active supply cylinder can be secured on a scale and then a manual switch can be made to the reserve cylinder as soon as a predetermined weight is reached. In the event that a CO_2 cylinder does run empty, the in-line gas-phase filters should be replaced.

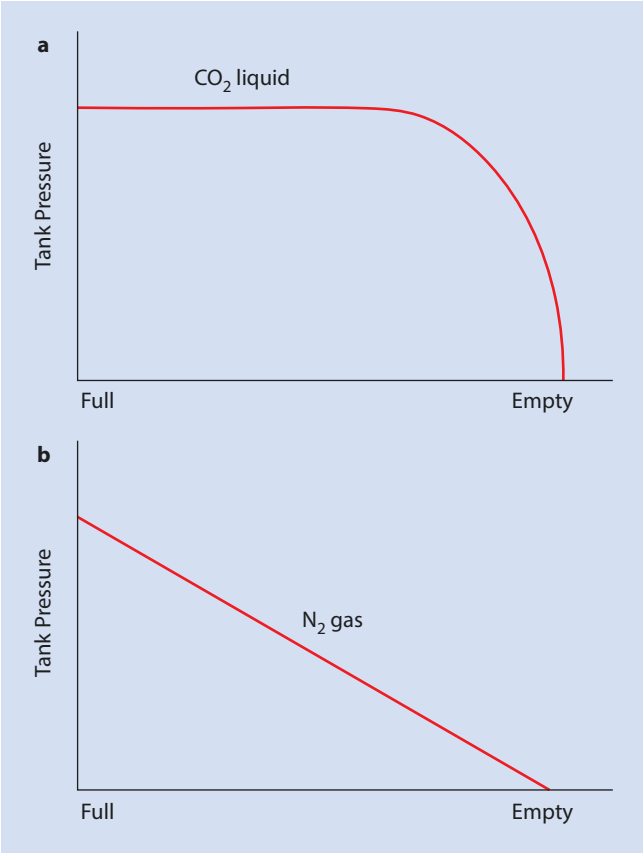

Fig. 10.3 **a** Because of the liquid state of the CO_2 supply and a constant pressure in the air space in the container above the liquid, one will not see a notable drop in manifold pressure until shortly before the cylinder runs empty. Liquid N_2 dewars will behave in the same manner. The manifold pressure is not an accurate reflection of the amount of gas left in the container. **b** In contrast, the pressure of compressed N_2 supplied in H-size metal cylinders is directly related to the amount of N_2 gas left in the cylinder, and the pressure in the tank is a reliable indication of the N_2 reserve

10.7.4 Oxygen

It is agreed that precise CO_2 control is essential for optimum IVF outcomes. However, the question must be posed if the same is true for O_2. It is possible that a slight variation in O_2 concentrations over time or a slightly elevated O_2 concentration can yield the same clinical results. Operating an incubator at 7% O_2 instead of 5% O_2 has significant implications on the amount of N_2 used, chamber humidity control, and the overall cost of operating reduced-O_2 incubators. Running out of N_2 may not have any direct short-term detrimental effect on the contents of the incubator, but depending on the brand of incubator and the type of CO_2 sensor, this may indirectly affect the CO_2 concentration and, therefore, the pH of the culture medium. Even though reduced O_2 in incubators have been correlated with improved clinical outcomes [13], concerns have been expressed regarding the detrimental effects of a possible too low O_2 concentration [2]. Similarly as for CO_2, the partial pressure of O_2 in the culture environment determines the amount of dissolved O_2 in the culture medium. Consequently, one should consider allowing higher concentrations of O_2 (6–10%) at higher elevations to ensure appropriate levels of dissolved O_2 in the embryo culture environment. Supplying N_2 to conventional incubators to lower the O_2 concentration can be expensive, cumbersome, and challenging. The cost savings, shortcomings, and benefits related to the gas supply of top-load, benchtop incubators have already been discussed. The N_2 needed for O_2 reduction can be provided as a compressed gas in H-size, medical gas cylinders, it can be siphoned off from larger liquid nitrogen supply tanks, or it can be generated continuously on-site by a N_2 generator.

Using compressed N_2 gas as the main source is the most inefficient and expensive, usually requiring daily tank changes. Changing tanks so frequently is labor intensive, demands significant space for an adequate supply of spare cylinders, and introduces daily variation to the IVF culture environment. Compressed N_2 gas is only as clean as the industrial manufacturing site. Considering that considerable effort and expense are generally invested in laboratory air quality and that approximately one-fifth (15% N_2, 5% CO_2) of the air in the actual incubator enters from the medical gas room, bypassing the laboratory air supply system, one should be diligent to apply the same standards of air filtration as for the laboratory room air. Nitrogen supplied to the incubators should be filtered for particles (HEPA filtration) and for gaseous contaminants (Fig. 10.4). The gas-phase filter should contain both activated carbon and permanganate (sodium or potassium salt) to ensure the filtration of both volatile organic compounds (e.g., hydrocarbons, aldehydes) as well as other common air pollutants (e.g., HS_2, SO_2).

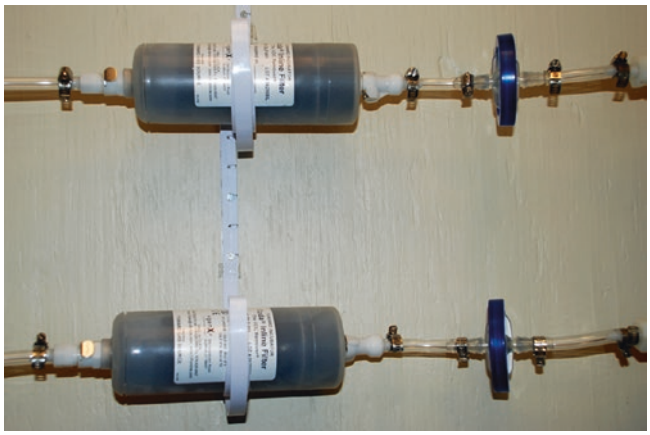

Fig. 10.4 All incubator supply gases should pass through a HEPA filter (particles) as well as a filter containing sufficient activated carbon/permanganate to remove gaseous contaminants

Siphoning pure N_2 gas off of 150 psi liquid nitrogen dewars instead appears to be a much more viable approach with changing of dewars required only every 2–4 weeks. In the liquid form, a typical dewar holds ~20 times more N_2 than an H-size cylinder filled with compressed N_2. A dewar can simultaneously serve as a liquid nitrogen source for IVF cryopreservation applications. As in the case with CO_2, the N_2 gas delivered from the evaporation of liquid nitrogen is pure and clean, provided that the dewar is not drawn from until empty. An ideal setup consists of a 150 psi primary and secondary dewar on a low-pressure manifold with high-pressure compressed N_2 gas cylinders as a tertiary backup.

Nitrogen can be supplied continuously to IVF incubators with the use of an on-site gaseous N_2 generator. With the atmospheric air consisting of roughly 78% N_2 gas, a N_2 generator only has to remove the other gases (O_2, Argon, CO_2, and others) to increase the percentage of N_2 to >99%. Atmospheric N_2 can be purified by membrane filtration (N_2 stays behind and the other smaller gas molecules pass through) or, alternatively, by pressure swing adsorption (PSA; smaller molecules are adsorbed on a pressurized adsorption bed and the N_2 passes through). PSA is the better technology yielding up to 99.999% pure N_2 gas. Not considering the investment cost of the N_2 generator, it is the most cost-efficient and least labor-intensive way to supply N_2 to IVF incubators. N_2 gas from compressed cylinders currently costs upward of \$4/100 ft^3; when from liquid N_2 dewars, upward of \$2/100 ft^3; and when generated on-site by an N_2 generator, roughly \$0.06/100 ft^3.

10.7.5 Humidity

After closing an incubator door, a significant volume of non-humidified N_2 is injected into the incubator to reinstate the lower O_2 concentration. This inadvertently will lower the RH in the incubator chamber for an hour or more after closing the door, especially for incubators with standard humidification pans. Therefore, the loss of RH after opening will be more pronounced in low-O_2 incubators compared with standard CO_2 incubators. As discussed under "Carbon Dioxide Measurement and Control" above, RH directly affects the CO_2 concentration in incubators equipped with TC CO_2 sensors, while the CO_2 concentration of incubators with IR CO_2 sensors is not affected. When using incubators with TC CO_2 sensors, it is, therefore, imperative to monitor and verify the RH on a frequent basis using an independent RH measuring device. It is not uncommon for inner doors or access ports to become less impervious over time with significant drops in incubator chamber RH.

10.7.6 Cleaning, Commissioning, and Decontamination

Incubators should only be cleaned when necessary without disabling the gas supply or switching off the power. Once completely switched off, it may take days, and sometimes weeks, to reach the same level of steady state as experienced before the shutdown. The CO_2 and O_2 sensors are particularly vulnerable when cleaning if the manufacturer's recommendations are not followed. The inside of the incubator can be washed with a soft, odorless soap solution to remove any culture medium and protein residue, followed by distilled water to remove the soap. Next, a 70% methanol solution is applied to remove oils and to assist with disinfection, followed by a second round of distilled water. Methanol is preferred over ethanol as a general IVF laboratory disinfectant because of its lower volatility index, typically releasing fewer fumes into the laboratory environment. The use of halogens such as bleach solutions (chlorine) or Betadine (iodine) is contraindicated as caustic agents to the incubator interior. Furthermore, halogen residues can persist in the incubator for an extended period to potentially affect future embryo cultures. Sterile, surgical gauze sponges are optimal for use when cleaning incubators. Shelves and water pans may be autoclaved; however, autoclaving glass inner doors is not recommended, since, over time, autoclaving tends to leave irremovable stains on the glass doors.

Starting up a new laboratory or introducing a new incubator into the laboratory, without compromising IVF outcomes, presents a special challenge. The concentration of gaseous contaminants found in a new incubator can be 100 times higher than that found in a seasoned incubator [24]. Off-gassing of new incubators can be enhanced by preheating the incubator chamber above normal operating temperatures for an extended period (days to weeks) before final commissioning for use. A thorough rinse of the inside of the incubator with a solution of household baking soda ($NaHCO_3$), followed by the standard cleaning protocol described above, further reduces odors from plastics, gaskets, and glues used during manufacturing. An intraincubator-activated carbon filter device may further improve incubator chamber air quality [25]. A new incubator should be at steady state for some days in all areas of interest (temperature,

humidity, CO_2, and O_2). Confirming a safe environment for IVF culture with a sensitive mouse-embryo bioassay is helpful. Even when passing all these quality control measures, some incubators may outperform others for no apparent reason [22]. Therefore, one should entrust only a few cultures to a new incubator over time and diligently and prospectively compare it with culture outcomes from an older proven incubator before commissioning the new incubator without reservation.

With good cleaning practices, attention to laboratory and incubator air quality, and sterile technique, contamination should be rare. Most of the new-generation air-jacketed incubators have sterilization cycles that can be incorporated into the regular maintenance routine or applied as needed. Sanyo and Galaxy incubators have an optional intrachamber ultraviolet lamp to complement chamber sterilization. Some incubators are manufactured with copper-stainless steel alloy interiors (standard for Sanyo) or with 100% copper linings, water pans, and shelves (options: Heracell, Binder, Galaxy) with inherent antifungal properties. Any observed or suspected contamination should be submitted for a culture and antibiogram. Knowing the identity and sensitivity of the organism can be crucial in identifying the source of contamination and to implement the appropriate corrective action. Contamination has been traced to room air, incubator air, incubator insulation, culture products, patient specimens (sperm or oocytes), and a lack of aseptic technique. Strategically placed bacterial and/or fungal culture plates can be helpful to identify the origin of contamination when air is suspected as a mode of transmission. Fungal contamination is by far the most common laboratory encounter, with *Aspergillus fumigatus* or *Aspergillus nigricans* a frequent isolate. When faced with fungal contamination and not equipped with high-heat decontamination capabilities, the chamber should be disinfected with 2% hydrogen peroxide, which is a proven fungicidal. Seventy percent methanol is not fungicidal. Some manufacturers provide decontamination kits (door seals, fans, filters) that should be installed after sterilization of the incubator chamber. A common source of fungal contamination is the interior insulation of older incubators. Insulation as the source can be verified by inspecting and culturing a small sample of the interior insulation. If confirmed, a contaminated incubator is best discarded. Furthermore, a review should be done of the general laboratory air conditioning system and concurrent humidity control as common contributors to fungal contamination. Daily inspection of the water pans can serve as an early warning sign of impending contamination. Water pans in the incubator function as a trap and reservoir for gaseous and particulate impurities and, therefore, should be changed at regular intervals. If changed too often, a continuous stable incubator environment will be harder to maintain. If not changed regularly, water pans themselves can become a source of contamination. Water pans should be autoclaved at a recommended interval of ~4 weeks and filled with distilled water or better to ensure chamber sterility and air quality.

10.7.7 Safety

Guidelines and requirements by most regulatory agencies with jurisdiction over IVF laboratories mandate that there should always be a backup incubator (minimum of two incubators per laboratory). Incubators should be monitored and the alarm system connected to an instant notification system 24-h per day when specimens are cultured. The number of incubators available should be adequate for the number of patients undergoing IVF at any given time. This will depend on the type of incubator used, the type of CO_2 sensor in the incubator, passive or active humidification, whether patients are grouped together for treatment, and other factors specific to each laboratory.

10.8 Discussion and Conclusion

Every laboratory operates under unique circumstances, and, in addition, personal preferences in equipment and in laboratory protocols should be considered. However, understanding the principles of incubator operation, realizing the shortcomings and strengths of various models, and being cognizant of available technologies are imperative for optimized IVF outcomes. With these in mind, the following guidelines should be considered:

- Culture in a reduced-O_2 environment.
- Consider top-load mini-incubators or the use of passive heat reservoirs for fastest temperature recovery.
- When using top-load incubators, be aware of the unique safety and quality control challenges posed.
- Conventional incubators equipped with IR CO_2 sensors are preferred. When having to use incubators with TC CO_2 sensors, humidity conservation is essential with thought given to the number of patients per incubator and incubator door openings.
- All supply gases (CO_2, N_2, premix) to the incubators should pass through a HEPA, activated carbon, and permanganate filter. Compressed N_2 should not be used as a primary source. When totally depleting the CO_2 or N_2 cylinders before switching to the backup source, the carbon and permanganate filters should be changed.
- Direct-heat air-jacketed incubators are preferred over water-jacketed incubators.
- As a rule, smaller incubators perform better than large incubators due to faster temperature and gas-phase recovery times.
- Calibrated, independent measuring devices should be used daily to monitor incubator chamber temperature, CO_2 and O_2 concentrations, and relative humidity levels. A case can be made to monitor O_2 and relative humidity only periodically.
- CO_2 settings should be determined based on pH measurements of the culture media guided by the pH target recommended by the medium manufacturer. Partial pressures of CO_2 differ with height above sea level, and therefore, different settings should be expected to achieve the same pH at different facility locations.

- A major preventative cleaning of incubators should be minimized and incubators switched off only if essential.
- Never use halogens for cleaning (bleach, iodine).
- New incubators should be burned in before use, according to a predetermined protocol, and appropriately commissioned through several quality control-guided steps.
- Refrain from allowing plastics, plastic test-tube racks, Styrofoam, glues, ink, and other aromatic substances in the incubator.
- Contamination should be rare and dealt with aggressively when observed. Fungal contamination is most common. Hydrogen peroxide can be used as a disinfectant for fungal contamination. Alcohols are not fungicidal.
- There should be ample backup incubators with a minimum of two incubators in any laboratory, regardless of type of incubator. Incubators should be connected to a 24-h alarm and notification system, monitoring all critical incubator parameters.

Review Questions

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1. Please describe the different types of incubators available.
2. Please define what the major functions of the incubators in the embryology laboratory are.
3. Please explain why low-O_2 concentration incubators are recommended for embryo culture.
4. Please describe how to perform quality control correctly on the incubators.

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What to Consider When Selecting a LAF, Class II Cabinet, or Isolette for Your ART Program?

Lars Johansson

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Learning Objectives

- To provide a quick overview of the products on the market
- To describe what you need to look for when selecting the best product
- To describe what products to buy that meet all requirements for gametes and embryos

11.1 Introduction

In order to construct and establish a high-quality assisted reproduction technology (ART) clinic, a multidisciplinary skilled task force needs to be formed that guarantees that all aspects concerning the design and building of the clinic, selection of equipments and disposables, as well as logistic treatment procedures are taken into consideration.

The clinic needs to be built in accordance with country-specific standards and legislations and perhaps also to international legislations, if cross-border treatments are also to be performed. The newly updated EU GMP guidelines and revised Annex 1 (tentatively published in early 2018) seem to suggest that ART clinics in the future might need an EU C-Class work environment (ISO 7), whereas an EU D-Class (ISO 8) is demanded currently. There will also be an evaluation of the effectiveness of the clinic's ventilation system (air change effectiveness index, ACE, and contamination reduction effectiveness, CRE), where perforated diffusers ensure an effective mixing of the inlet air and a cleaner environment.

In addition, the clinic must proactively prevent the exposure of oocytes and embryos to environmental embryotoxic pollutants from within the clinic (building materials, ventilation, laboratory furniture, light and gas source, disposables, detergents and cleaning agents, clothing materials, and bio-burden of staff and therefore restricted access to the laboratory [1–8]) and ensure that the couple's gametes and embryos are given an optimal chance to develop into top-quality embryos with high implantation and low miscarriage rates [9, 15].

This chapter tries to give information and guidance in the selection of a suitable unidirectional airflow (UDAF) bench, Class II-III Cabinets, multipurpose workstations, or isolettes that meet your needs in terms of quality and costs, operational features and options, service and support expected by the companies, legislation within your country, expectations from the couples for optimal handling of oocytes and embryos, identification, and safety. This chapter focuses on the most frequently used brands on the market, but there are both regional differences in preference of brands and availability of additional products. (Tables 11.1 and 11.2).

The building materials of the workstations should be of the highest quality, preferably antibacterial, easy to keep clean, resistant to cleaning detergents and disinfectants, impervious, seamless, and non-embryo-toxic, and must be given time for outgassing and function tests (particle and microbial count) before it is taken into usage.

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Table 11.1 Regional differences in preferences for UDAF benches

Americas	Europe	Asia/Pacific
K-Systems	K-Systems	ESCO
IVFTech	IVFTech	IVFTech
	CooperSurgical	K-Systems
	ESCO	Astec

Table 11.2 Characteristics of different types of UDAF benches

Characteristics	K-Systems	IVFTech	ESCO	CooperSurgical	Astec
Sizes (mm)	1200, 1500, 1800	900, 1200, 1500, 1800	1200, 1800	900, 1200, 1500, 1800	1000, 1300, 1600
Filter	HEPA, carbon	HEPA, carbon	ULPA	HEPA, carbon	HEPA
Noise levels (dB)	<51	<51	<47	<48	?
Electric stand	Yes	Yes	Yes	No	?s
Ergonomics	Flat front	Sloped front	Flat front	Sloped front	Flat front
Light source	LED optional	No LED	No LED	LED standard	No LED
Heated surface (mm)	Electric, three zones	Electric, one zone, Corian	Electric, nine zones,	Electric, one zone	Electric, one zone
Heated glass stage	Standard, 90	Optional	Standard	Standard, 90	?
ICSI station	1200, 1800	Yes	No	1200, 1800	?
Built-in incubator	Yes	Yes	Yes	Yes	?

All laboratory staff must also be educated and their competency tested in the operation of these equipments (technical standard operational procedures), treatment procedures (SOPs), and quality controls [10, 11]. Each piece of equipment should be labeled with a chronological identification number, and repairs, annual services, and action taken must be registered. The most sensitive and vital equipments must be connected to uninterrupted power supply (UPS) to avoid disruption of work, recalibration, and damage to the equipments or gametes and embryos.

11.2 Principles of Different Types of Workstations

11.2.1 LAF (Laminar Flow) or UDAF (Unidirectional Airflow) Benches

IVF-Tech – ► www.ivftech.dk; Origio and K-System – ► www.coopersurgical.com; Astec – ► www.astec.com; Kojair.- ► www.kojair.com; ESCO – ► www.escoglobal.com; Telstar – ► www.telstar.com; EuroClonegroup – ► www.euroclonegroup.it; LAFTech – ► www.laftech.com.au; Gelman – ► www.gelmansingapore.com

The vertical laminar flow UDAFs are the most commonly used workstations within ART. Unfortunately, and due to the cooling effect of the airflow over the content of the culture dishes, the clinics run the UDAFs at half ventilation speed, which corresponds to only 15% of their efficiency. Thus there is no guarantee that the work area is kept at the needed standards for ensuring that the patient's culture dishes are not contaminated during different procedures.

The benches should run continuously since this also will contribute to the cleanliness of the laboratory. The personnel must know that if a bench is switched off and started up again, it will initially (15 min) release a substantial amount of debris that will contaminate everything within the bench. All this can be avoided if programmable benches are bought where turn off and on times can be programmed so that all parameters (temperatures and cleanliness) are at optimum when the work starts in the morning. However, most UDAF benches I have seen in the clinics are overloaded with items that affect the cleaning efficiency of the bench. Behind each object turbulence is induced that is three times the width of the object, where particles are precipitated, which increases the contamination risk.

The benches are usually equipped with a heated surface that might give an equal temperature over the whole surface area, a heated glass stage, and a light source. A high-quality stereomicroscope should be used for search and handling of COCs, evaluation, and selection of embryos for embryo transfer and vitrification. A humidified pre-warmed gas support and oil overlay of the culture dishes ensure that the humidity, osmolality, and pH of the culture media are kept within expected optimal ranges. Another option is to have an integrated incubator at the back wall of the workstation, but be aware that these types of incubators also have a very long recovery time of the culture conditions after one door opening. Thus rather use the built-in mini-incubator with a proven quick recovery of the culture conditions.

In the double LAF benches, each side is equipped with a separate control system for heating, humidity, and light; thus, it can therefore be used for other purposes like vitrification or for testicular biopsies. However, there are also multi-zone benches where the temperature of each zone is individually adjusted and under surveillance.

Benches or workstations can also be used for ICSI, IMSI, and biopsy techniques if an inverted microscope equipped with a laser and micromanipulators is installed on an anti-vibration table. However, be aware that vibrations might affect the outcome of the procedures.

11.2.2 Class II/Safety Cabinets

11.2.2.1 Most Companies Selling LAF Benches Also Sell Class II Cabinets

The safety cabinet protects both the product and operator, which regulatory bodies in some countries require. However, these cabinets are mostly used in a separate laboratory for handling and preparation of semen samples and especially for handling samples from patients with infectious diseases.

The very high airflow within these cabinets affects the temperature of the dishes and can simultaneously also alter the pH. This makes the Class II bench less suitable for handling of oocytes with temperature-sensitive meiotic spindles that might become disrupted, which later affects the embryo quality.

11.2.3 Class III Safety Cabinets (Isolators or RABS (Restricted Access Barrier Systems))

Closed systems (isolators or RABS) are usually used for processing aseptic products, toxins, and biological materials where contaminations and/or cross-contaminations must be avoided. These types of equipments are mostly used in the pharmaceutical manufacturing industry.

There is a physical barrier between the staff and the work area within the isolator. Access into the isolator is via sealed port glove-sleeve assemblies.

11.2.3.1 Mobile Chambers, Isolettes or Smart-Stations

G-603 (K-System, ► www.coopersurgical.com); Cell-Tek 3000 Series (► www.Tekevent.com); SmartStation SS250 (► www.Astec.com); UNICA (► www.IVF-Tech.com), SCE-IVF (► www.escoglobal.com).

These mobile and enclosed workstations, with an incubator-like environment and fitted with a suitable stereomicroscope or TV monitor, are suitable for search of cumulus-oophorous-complexes (COCs) in follicular fluids

during retrieval, evaluation, and selection of embryos for ET, denudation of oocytes, and microtechnologies (ICSI, IMSI, and PGD). The stress on gametes and embryos is minimized since the inner environment is controlled and adjusted for pH, osmolality (humidification of the environment via heated water reservoir), and the temperature of the work area [2]. The heated plate can independently be turned on or off and adjusted to various laboratory procedures.

There are also possibilities of having built-in mini-incubators and pre-warmed dishes, thus less handling of oocytes and embryos outside the workstation.

HEPA, carbon, and VOC filters or UV photocatalytic removal of VOCs provides the workstation with a clean room environment. However, it is still advisable to use MOPS- or HEPES-buffered media, with an oil overlay, during aspiration, handling, and injection of oocytes (ICSI). Such dishes ought to be stored under the lid of a separate heated gas purge inlet hole, when they are not used and for a better control of the pH, since workstations are not leak proof.

The work area is accessed via small holes in the front of the workstation, and side openings are for introduction of dishes, test tubes, heat blocks, and aspirated follicular fluids or removal of waste.

These mobile workstations can be fitted with a motorized height-adjustable stand on which small gas cylinders can be mounted. The content of the gas cylinders can either be mixed by the workstation itself or pre-mixed gas cylinders could be used.

Since these workstations are fitted with lockable large rubber wheels, they can easily be relocated between different rooms, provided that different kinds of contaminations are not also transferred.

There is also the matter of decontamination and cleaning in closed workstations. In open workstations the cleaning of any spillage is straightforward, whereas in closed workstations it is more complicated.

11.3 Basic Constructions and Comments

11.3.1 Steel Frames, Stands, Sloped Fronts, and Side Walls

The benches or cabinets usually come with stands of fixed heights, most commonly 700, 800, or 900 mm; they might have a footrest and perhaps a practical solution for integration of a computer or storage shelves underneath the workspace area.

There are also electrically operated height-adjustable (750–1110 mm) stands so the staff can either be seated or work standing. A good investment, for the electrically height-adjustable stands, is a sensor on top of the bench or cabinet that alarms when it comes too close to the ceiling. Many manufactures do not sell the electrically height-adjustable stands since they can become unstable and cause accidents. If an electrical stand is bought it should have been developed according to EN 61010 and assure full stability at its highest elevation. Most of the benches come with leveling feet and/or wheels and are 900, 1200, 1500, or 1800 mm in width.

A sloped front allows an ergonomic, fatigue-free, and comfortable work environment and has usually a programmable LCD display control system in easy reach for the staff. The side walls can be of stainless steel, glass, or polycarbonate, and they can also be equipped with pre-drilled holes.

A sliding shelf, under the surface of the LAF bench, is an efficient way to store a keyboard and to keep the workspace clear.

11.3.2 Filters, Motors, and Noise Levels

Most benches come with thick HEPA (99.99% efficiency in removing 0.3 um particles) or ULPA (99.999% efficiency in removing 0.1–0.3 um particles) filters of a high quality that do not easily block and can therefore be used for a very long time before the filter must be exchanged. There is therefore no requirement for large strong fans that generate high noise levels and vibrations, thus a good work environment.

Some benches have also built-in dampers that furthermore reduce vibrations and facilitate evaluation of oocyte maturation, fertilization, and embryo quality and other sensitive microscopic work.

The prefilters on top of the benches are easily exchanged, and the large HEPA/ULPA filters should be easily exchanged when the airflow monitoring system indicates it. When a new bench is installed, the distributor must ensure that the filters are not damaged during the transport. Some benches can also be fitted with activated carbon filters for partial removal of volatile organic compounds (VOCs).

11.3.3 Stainless Steel or Corian Table Tops

The most common work zone consists of a single piece of stainless steel but can also be made of Corian (HiMacs). In some benches, the surface has been coated with a silver-ion-impregnated antibacterial/antimicrobial coating (Isocide) that prevents growth of bacteria and reduces the risk of contaminations.

The absence of screws or connectors in work zones and rounded easy clean corners in the bench reduces potential contamination and facilitates the maintenance and cleaning of the surface. Cleaning is performed with special embryo-tested cleaning detergents at the end of the day. However, in between patients and if spillage occur, the spillage should first be removed; the surface washed with water for injection and finally cleaned with a detergent. If the detergent makes the surface sticky or greasy, clean it again with water for injection.

11.3.4 Heated Surface and Glass Stage

Select a heated work area that meets the requirements for the number and types of treatments. Most of the clinics

equip their benches with an electrically controlled heated surface since water-controlled heating needs more attention and might also become a source of contamination of the cultures. In the multi-zone ART workstations, each zone is independently controlled (accuracy: ± 0.2 °C) and surveyed. The surveillance system provides the user with real-time information of the different zones' performance as well as parameters for gas pressure and flow rate. Ensure that there is no crossover heating between the different zones.

Note: Remember that the temperature of all heated surfaces of the bench must be calibrated with a sensitive temperature probe within the most commonly used dish and not against the surface of the bench! [16]

The pre-mixed gas pipelines, embedded in the electrically heated sheet underneath the heated surface, are connected to a heated and pressure-controlled humidifier providing dishes with filtered humidified pre-mixed gas.

The heated glass stage has a dedicated and uninterrupted power supply to further strengthen the temperature control of this very important area where the staff searches for COCs in follicular fluids, performs denudation of oocytes, evaluates fertilization, controls the quality of embryos, and selects embryos for transfer. The glass stage must be in level with the heated surface area to avoid relocation or loss of oocytes or embryos.

There is also a need for an easy access for service staff and laboratory personnel to gas pipelines, electric cables, and light fittings.

11.3.5 Gas Support, Filters, and Humidification

In order to maintain optimal culture conditions within the dishes during egg collection and handling of embryos, the benches are fitted with a pressure-controlled and humidified gas support system. The connecting gas tube must be of nontoxic and VOC-free material, leak proof, and easily fitted to the gas reduction inlet valve. The concentration of CO_2 must be adjusted to that of the clinic's choice of culture media and contain low oxygen (5–6%), which ensures the development of high-quality embryos of high implantation rates.

The compressed gases may contain high levels of organic contamination. It is therefore essential that the gas quality should be of the highest purity (medical grade for both nitrogen and carbon dioxide) and have the lowest concentration of contaminations [3, 12].

The benches can also be fitted with a gas mixer that mixes the CO_2 and the N_2 to recommended concentrations for optimal outcomes.

Many clinics also pre-clean the inlet gas via an inline HEPA, charcoal, and potassium permanganate filter before it is heated and humidified.

Take care to select a bench where the heated surface gas inlet is as close as possible to the stereomicroscope so the work is facilitated. The companies usually also supply glass hoods which are positioned over the gas inlet hole and the culture dishes. Thus, the cultures are kept at an optimal pH via the pre-warmed humidified gas mixture. The humidifier must be regularly cleaned with nontoxic detergents that do not leave any residue behind, sterilized, and filled with water for injection.

11.3.6 Pass-Through Hatches/Tunnels and Built-in Incubator(s)

Some of the benches can be provided with a temperature-controlled and calibrated pass-through hatch or tunnel with direct access to the egg collection room. Test tubes containing aspirated follicular fluid are placed in the heated blocks by staff in the egg collection room and their content evaluated by the embryologist in the laboratory.

Also, single door incubator can be installed in the back wall of the bench. Since the incubator door is frequently opened, the culture conditions are never optimal, which affect the outcomes of the treatments. It is better to use built-in mini-incubators, with individual small doors and a gas inflow purge, for a quicker recovery and better maintenance of the culture conditions. However, also control the lids of the dishes for condensation, since that is an indication of evaporation and problems with the osmolality of the culture media. The latter can easily be avoided by using oil overlay of the culture media in the dishes, which most clinics also practice.

Yet another option is to transfer the dishes, after completed work, from the glass hood to mini-incubators, close to the bench and with a quick recovery of the culture conditions. Only use large incubators for pre-incubation of culture dishes and not for cultures. Very recently, some models of IVF workstations have come out, which provide inbuilt small-bench top incubators integrated to the work surface.

11.3.7 Stereomicroscopes, Light Source, and High-Resolution Screens

There are a lot of different stereomicroscopes that are suitable for the work within the benches. The stereomicroscope needs to be fitted with an adjustable ergonomic tri-ocular tube so also a good camera can be connected to a TV monitor, within or outside the bench [4]. The space between the heated light source and the objective should allow easy handling of dishes, oocytes, 2PNs, and embryos, so I recommend a 0.7× objective.

The combination of a high-quality stereomicroscope, a camera and a light source, where the mirrors can be angled and adjusted (diascope light), and the generated picture evaluated on a high-resolution screen makes it even possible to evaluate pronuclei. For this, it is very important that the bench has dampers so vibrations are avoided. The new light

sources are fitted with LED light, thus longevity and less change of bulbs.

Any cables are led through the pillar of the stereomicroscope.

It is an advantage to have the high-resolution screen on the outside of bench for dual control, evaluation, and teaching purposes. It also makes it much easier to repair a screen that is located on the outside versus a screen mounted within the UDAF bench.

In addition, there are touchscreen monitors available from which you can view, record, and export images of gametes and embryos. A built-in joystick in the bench controls focus, brightness, contrasts, and magnification of the digital and inverted microscope.

11.3.8 Optional UV vs UV-C Sterilization

The detoxification or rather the removal of pollutants is very important in IVF clinics since they could have an enormous impact on embryo quality, embryo survival, and thus the clinical outcome of the treatment.

Many pollutants settle on work surfaces or into tissue culture plastics where they could dissolve in the culture media. Therefore, some UDAF benches but mostly Class II Cabinets can be equipped with time-regulated UV lights for sterilization of the work areas. A built-in controller monitors the effectiveness of the UV light and when it needs to be replaced.

UV light is divided into three different sources: UV-A 315–400-nm, UV-B 280–315-nm, and UV-C 100–280-nm wavelength. The UV-C wavelength of 254 nm effectively destroys bacteria, viruses, and VOCs (volatile organic compounds) and does not produce the very toxic ozone by-product [5, 13]. Ozone in itself is a highly effective decontamination product for the lab surfaces and HVAC system, but not suitable for IVF clinics since it is highly unstable and reactive.

All higher organisms are protected from low exposure to UV radiation by their external skin or cortex. Since gametes and embryos do not have these kinds of protective layers, they are more vulnerable to exposure of ozone.

11.3.9 ID Control

(Matcher, ► www.imtinternational.com; RI Witness, ► www.riwitness.com)

There are at least two electronical witnessing and traceability systems created for IVF clinics and donor banks. The aim is to prevent errors in identification of patients, their gametes and embryos, as well as products used during their treatments. These electronic witnessing systems provide a double control system that ensures that all procedures are performed in a chronological manner with patient-customized name and barcoded lab ware and media products or via radio frequency identification (RFID) technology [6, 14].

The identification system is installed within or on the surface of the UDAF (LAF) benches and Class II Cabinets.

11.3.10 Inverted Microscope

Some companies also provide workstations where an inverted microscope has been integrated on a free-standing antivibration table. The latter prevents vibrations from the UDAF bench reaching the microscope, and hence the staff simultaneously can work aseptically during ICSI, IMSI, and biopsy procedures. This also provides better mobility of gamete−/embryo-containing dishes in lesser time between ICSI and IVF workstations.

11.3.11 Lighting

All light fittings are recommended to be of yellow light with low VOC emittance and made out of top-quality components.

The light tube should be positioned so it can easily and safely be replaced when needed.

11.3.12 Service Agreement and Spare Parts

It is advisable to get at least a 2-year service agreement included in the purchase deal to avoid any unforeseen extra payments due to hidden problems.

If you need service will it be undertaken by educated service staff in a timely manner and do they have spare parts available within your country? Or do you have to pay for expensive repairs from abroad?

Chronologically label all equipments and have a separate register over repairs for each piece of equipment in order to document and detect “hidden” problems.

11.3.13 Daily Routines and Quality Controls

All UDAF benches and Class II Cabinets should continuously work since they also keep the laboratory environment clean. Every time they are turned, there is a release of particles that contaminates all items stored within the benches.

After a day’s work, the surface area is cleaned with approved cleaning detergents following the prescribed cleaning routines. Also control the surface underneath the bench, or the Class II Cabinet, since splashes from waste products (follicular fluids, semen, blood, etc.) might have contaminated it.

The following morning and before any handling of gametes or embryos, perform a routine control of the functionality of the equipments, either via a wireless system or via manual documentation. Also the function and settings of stereomicroscopes (Köhler) and inverted microscopes must be performed and registered.

11.4 Where to Place the UDAF Benches or Class II Cabinets?

The floor to ceiling height of the laboratory, the location of ventilation inlets and outlets, UPS-supported electrical outlets, and pre-mixed gas reduction valve must be considered when you decide where to place the UDAF bench or Class II Cabinet.

It is important to see to that you have enough of electrical outlets so that cords are inserted directly into the outlets without any extension cord. All outlets should be fitted so they reside within the wall, which prevents the collection of dust that potentially over time could contribute to contaminations within the laboratory.

The pre-mixed gas reduction valve is for built-in mini-incubators or mini-incubators next to the UDAF bench or if the UDAF bench is provided with pre-mixed gas inlet hole(s) for gassing of culture dishes.

After extensive cleaning and "burn-off" of toxic volatile organic compounds (VOCs) in another nearby room, it is placed and leveled in its final position in the laboratory. Thereafter, the staff undertakes a comprehensive training program in the handling and maintenance of the bench (Written technical SOP) via the company that you purchased it from.

11.5 Cleaning and Disinfection

Before any measurements of release of particles from the filters within the bench or cabinet and biological contaminations, the equipments must undergo a thorough cleaning and decontamination. All parts are pre-washed with water for injection for removal of potential contaminations and followed by disinfection by approved ART products like Oosafe. Avoid 70% ethanol since it only reduces and does not eliminate the contaminations, and hydrogen peroxide might damage the metal parts of the equipment over time.

The daily cleaning routines are preferably performed at the end of the day (usually in three steps (water, Oosafe, and water)) so you avoid cleaning in the morning and the release of potential toxins from the detergents. Since the bench or cabinet is constantly running, all fumes from the detergents are eliminated overnight, provided the equipment is serviced and controlled yearly. Remember to register all services and repairs for each piece of equipment.

Any spillage in between patients should be immediately cleaned with dry tissue or tissue wetted with water for injection only. Only clean it more thoroughly when no oocytes or embryos are to be evaluated or manipulated.

"Do not forget the effects of personnel bio burden on the environment and the outcome. The dress code must be followed in all aspects and the staff must understand why jewelries, mobiles, long nails, nail polish and scented products (cologne, perfume, deodorant, hairspray, facial foundation, body wash or powder, after shave, etc.) cannot be used. Smoking staff members should only work in the andrology or sperm preparation laboratory, since tar particles within their breath and contaminations of skin and hair are released for a prolonged period of time after smoking." [7, 15]

11.6 Quality Control (QC) and Calibration

Provide the equipment with a specific chronological identity and bar code, which is used to register all parameters (QC), services, yearly recertifications, reasons for malfunctioning, and repairs.

After extensive cleaning and decontamination, the equipment is controlled for the release of particles and biological materials (bacteria), which is more frequently done during the upstart period to reveal malfunctions. The airflow within the bench/cabinet is evaluated with a smoke test, which visualizes airflows.

The heated surface temperature and the heated light source are calibrated within the most frequently used culture dish, at a specific fan speed, with a surface temperature probe. The final surface and light source temperature should be within 36.5–36.9 °C for best outcome, which also causes less damage to the most sensitive stage of the oocyte – the meiotic spindle [8].

As for the surface of the workstations, the heated glass plate, on the inverted microscope, is also calibrated to 36.5–36.9 °C, with a surface temperature probe, in a flat ICSI dish. Ensure that the bench/cabinet does not cause vibrations of the injection pipettes, which potentially could increase lysing rates of injected oocytes.

For all the above, it is better to purchase all equipments from your local dealers since it simplifies communication and service.

11.7 Written Standard Operational Procedures (SOPs)

Standardization in handling of the equipments and ART procedures makes it easier to improve techniques or test new products. It gives traceability of all actions performed, makes the processes and procedures more efficient, and improves service quality, which is beneficial for both customers and staff.

SOPs are upgraded yearly and the latest international technologies and bench mark processes and procedures are incorporated in a never-ending project.

The competency of the staff in different techniques and procedures is tested yearly and saved in their personal file.

The traceability of relevant data related to products and materials (batch number and expiration date) that come in contact with the patient's gametes or embryos during their treatment cycle is recorded in the patient's file and a log book.

By the use of key performance indicators (KPIs), the clinic detects changes in results, performance of staff members, and areas in need of reeducation. Indicators should be internationally defined and recognized, which reduces misinterpretations and facilitates internal as well as international communication and auditing [9, 17].

11.8 Evaluation of the Performance of the Bench/Cabinet

Before any treatments are performed in the new bench or cabinet, all measured parameters should be within the set threshold values. All staff members should also have been properly trained and their competency controlled. Select a number of suitable couples where you expect a high number of oocytes after egg collection, denudation or micro-injection, and embryos for evaluation and embryo transfers. Perform a sibling study against another bench or cabinet and compare the ease of work and outcomes.

11.9 Final Comments

The position of the cabinet can have a major effect on its performance, and it can also affect or become affected by the surrounding equipments, walls, benches, laboratory through traffic, and of course the air handling system. Care must be taken to ensure that the inflow diffusers are positioned so they do not create turbulence across the cabinet inflow area.

Even if the clinic has invested in high-quality UDAF benches or cabinets and controlled their performance, the outcome of the treatments is also very much dependent upon how the treatment is performed. For instance, the disposables selected by the clinic must have shown to consistently give good results in clinics all over the world. The choice of disposables is the preference of the clinic as long as they have been embryo-tested with several different methods and are non-pyrogenic, preferably gamma-irradiated, and of clear plastics, facilitating inspection and evaluation of gametes and embryos. Pipetting of culture media is performed according to best practice and with sterile filter tips of high quality (RNA, DNA, and pyrogen-free). The plastic covers might be toxic so there is a need for fuming off of all particles on the surface of the UDAF bench or Class II Cabinet before they are used. To avoid contaminations, all handling of the culture media is performed with embryo-tested powder-free sterile gloves without chemical additives, accelerants, or emulsifiers.

Review Questions

1. Why is it important to have perfect control of the temperature during egg collection, denudation of oocytes, and evaluation of embryos?

   The meiotic spindle in oocytes is very sensitive to fluctuations in temperatures, which can cause aneuploidies and adverse effects on the embryo development. Variations in temperatures can also cause developmental problems within embryos and lower implantation rates.

2. The engines of the ventilation system must be silent and not cause vibrations! Why?

   Working in benches or a laboratory with equipment's generating high noise levels give discomfort to staff and could also affect the work, if micro-manipulation techniques are implemented in the LAF/Class II.

3. A high-quality light source and a reliable temperature-controlled heated glass is one of many important features of a high-quality UDAF bench! Why?

   Unreliable light source will affect the search for COCs, oocytes, and handling as well evaluation of embryos. Defect temperature control could affect the meiotic spindle, embryo development, and outcomes (IR).

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Culture Media in IVF: Decisions for the Laboratory

Jason E. Swain

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Learning Objectives

- Identify key energy substrates included in human embryo culture media and the developmental stages that utilize them
- Discuss macromolecule supplementation and available options in clinical IVF
- Understand pH control of embryo culture media and factors within the laboratory that impact pH

12.1 Introduction

Substantial efforts have been taken in attempts at refining and improving IVF culture media since the initial reports of human embryo culture utilizing somatic cell media, such as Ham's F10 [1, 2]. Through the work of pioneers like Quinn and Menezo, media designed specifically for human embryo culture in vitro initially became available [3, 4]. Extensive studies in the mouse from the lab of John Biggers led to media formulations later adopted for use in clinical IVF [5–8]. Subsequent modifications and refinements to these media ensued, and it is now possible to routinely grow blastocysts in vitro in defined media without the use of serum.

This pursuit has resulted in an extensive body of literature examining various culture media formulations, culture paradigms, supplemental additives, and their impact on embryo development. Because of the breadth of these data, an exhaustive review of all media components is beyond the scope of this chapter. However, certain topics are required for embryologists to gain appreciation for the progression of the field, to understand the rationale for current media formulations, as well as to have the ability to make informed choices when it comes to selection and use of commercially available media within the laboratory. Importantly, certain aspects of IVF culture media, such as selection of media type, pH set point, and macromolecule supplement, are still directly decided or controlled within the IVF laboratory. Furthermore, media characteristics such as pH and osmolality can be inadvertently altered in the laboratory and thus require special attention. Review of these topics is necessary to optimize laboratory efficiency and ultimately IVF outcomes.

12.2 Laboratory Choices and Culture Media

12.2.1 Manufacturing and Consistency

Commercial production and supply of various IVF culture media is one of the primary causes for dramatic improvements in the field. Utilization of ultrapure water, clean facilities, and impeccable quality control during production has helped reduce much of the variability previously encountered within the IVF laboratory.

12.2.2 Media Formulations

Formulations should consider the specific cell types involved. When one mentioned "culture media" in the context of the IVF laboratory, this can actually refer to an entire suite of products that are often formulated based on the specific needs of the cell type for which use is intended. Sperm, oocytes (cumulus enclosed vs. denuded), and embryos of various developmental stages have different nutritional needs and environmental sensitivities. Thus, consideration of these cell types and the associated procedural steps should be considered when selecting media, and the multitude of media used throughout the IVF process should be considered when attempting to discern impacts of "culture media" on embryological or perinatal outcomes.

Most often, focus is placed on the medium used to culture preimplantation embryos.

In the last 15–20 years, there was a dramatic shift toward the use of sequential culture media, where cleavage-stage embryos are cultured in one medium formulation for the first 1–3 days and subsequently placed into a second medium formulation for the next 2–3 days to support morula compaction and blastocyst development. These media vary slightly in their composition, changing the levels of glucose and other energy substrates as well as altering the complement of amino acids, chelators, and other components. The rationale behind this approach is to meet the changing needs of the embryo by attempting to emulate what is observed in vivo, where the composition of oviductal fluid differs from that of uterine fluid [9]. In addition, this approach entails replenishing embryos with fresh culture media, ensuring adequate supply of substrates and removal of potentially harmful by-products, such as ammonia (Fig. 12.1).

In contrast, monoculture systems entail supplying a single medium formulation that is a product of exhaustive experimentation designed for "letting the embryo decide" which components it prefers [10]. These media have received renewed attention, largely due to the increased implementation of time-lapse incubators and the ability to use with uninterrupted culture throughout the culture period in attempts to minimize environmental perturbations. The approach of replenishing media at 2- to 3-day intervals to supply fresh nutrients and remove waste products is also compatible with this approach. Indeed, in a number of laboratories, monoculture systems prove to be as efficient as sequential culture systems in terms of embryo development, morphokinetic timings, and implantation/live birth outcomes [11–18].

Extensive data on the release or depletion of nutrients by both mouse and human embryos led Leese [19] to propose the "quiet embryo hypothesis," which suggests that a reduced metabolic level is consistent with a viable phenotype. Further, environmental stresses are seen as one of the components that elevate metabolic activity resulting in heritable damage to daughter cells

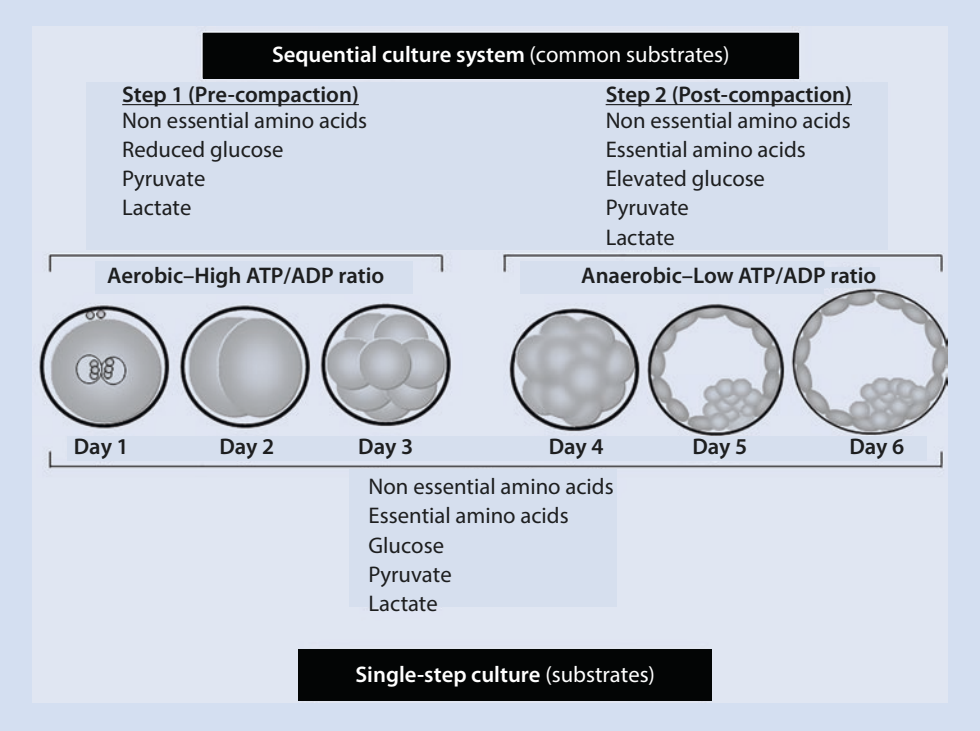

Fig. 12.1 Key energy substrate components of embryo culture media

such as heat shock and nucleic acid damage [19, 20]. The extent to which "noisy metabolism," induced by stresses from the in vitro environment, influences embryonic responses to various metabolite challenges during medium design experiments remains unknown. But it is important to realize that all current media formulations were developed in animal models, not with human embryos, and it is not known that human zygotes respond in the same manner as those from other species to a specific environmental challenge. Medium design should therefore be considered a work in progress.

Regardless of the approach employed, the success of such a wide variety of media underscores the plasticity seen with regard to the embryo's ability to adapt to its environment. Importantly, though many media support embryo development, their efficiency needs to be evaluated in the context of their use within a particular laboratory, in conjunction with the variety of other supplements and processes that make up the complete culture system [21, 22]. To this end, careful attention to detail and development of more sensitive endpoint assessments are needed to truly tease out minor improvements and lab-to-lab variability. Emerging data indicate that "embryo culture media" may impact human embryo gene expression patterns [23, 24] and perhaps even birthweight [25–29], though existing studies have done a poor job in controlling for confounding variables in the culture system that may also impact results (i.e., protein supplement, pH, oxygen tension, etc.) and fail to isolate culture media as the sole variable potentially responsible for observed outcome differences. Others indicate that media have no impact on perinatal outcomes [30, 31]. Independent of which media are utilized, certain themes hold true and are important to understand in order to appreciate intricacies of embryo physiology and to assist in making informed decisions within the lab.

12.2.2.1 Glucose

Glucose has been a wide source of contention within the field of embryology and is one of the primary variables in sequential culture media. Though cleavage-stage human embryos can use glucose, it is in minimal amounts compared to oxidative substrates like pyruvate [32–35]. Indeed, sequential systems utilize reduced levels of glucose (0–0.5 mM) in the first step of their system, also often include EDTA, which further suppresses glycolysis [36]. Furthermore, culture media devoid of glucose support high rates of human embryo development and pregnancy during the first 3 days of culture [37–40]. Thus, any minimal initial requirement for glucose may likely be obtained through initial exposure and carryover from handling media containing glucose or from internal stores of glycogen. Metabolic studies indicate that mammalian embryos primarily utilize glucose during compaction and blastocyst development. Therefore, the second media employed in sequential systems raise the concentration of glucose (>2.0 mM). Single-step media supply glucose at intermediate levels and also contain EDTA. This would seem to contradict the rationale used to rationalize the need of sequential media but, in the context of other nutrients, is able to support embryo development to the blastocyst stage.

12.2.2.2 Lactate/Pyruvate

Though extensive focus has been placed on glucose and its role in supporting blastocyst development, perhaps more important are the roles of energy substrates that permit the prerequisite cleavage-stage embryo development. The addition of lactate to early salt solution media was instrumental in supporting development from the two-cell stage of mouse embryos [41]. Furthermore, pyruvate was discovered to be imperative for cleavage of one-cell mouse zygotes and is the

primary energy substrate for cleavage-stage embryos [42, 43], including human, where it is used throughout development to the blastocyst stage [34, 35, 46]. Importantly, low levels of lactate can affect pyruvate metabolism [47, 48] and can also acidify internal pH [50, 51]. It has been suggested that only the metabolizable L-lactate isomer be included in culture media formulations to avoid excess suppression of internal pH that may exist with the use of D/L-isomers [47, 48].

12.2.2.3 Amino Acids

Amino acids can serve as energy sources to developing embryos but also serve to maintain intracellular homeostasis by acting as zwitterions to buffer pH and antioxidants and as organic osmolytes to control cell volume [52]. As a result, amino acids are now an essential component to successful embryo culture media. Tedious sets of experiments were conducted examining effects of individual amino acids on hamster embryo development [53]. These studies demonstrated that three amino acids in particular improved development in the context of the utilized base medium: glutamine, taurine, and glycine. Additional experiments conducted by Lane and Gardner utilized commercially available mixtures of amino acids known as Eagle's "essential" and "nonessential amino acids" in a sequential culture system. These studies determined a preferential exposure sequence that improved embryo development and viability following transfer, which entailed addition of nonessential amino acids with glutamine during the first 3 days of culture, followed by inclusion of all 20 amino acids during the following 2–3 days [54]. Benefits of amino acids are also evident when included in a monoculture system, such as KSOM, yielding improved blastocyst development, cell number, and hatching rates [6, 8, 55]. Indeed, even brief exposure of mouse zygotes to media lacking amino acids can impair development [56]. Therefore, it is imperative that all media contain some source of amino acids, especially handling media utilized for oocyte retrieval and ICSI.

One potential concern with amino acids in culture, especially during cleavage-stage development, is their breakdown and production of ammonia, which may impact subsequent development and health of the resulting offspring [57–61]. Of particular interest is glutamine, which is the most labile of the amino acids [62, 63]. Fortunately, dipeptide forms of glutamine, such as alanyl-glutamine [59]) or glycyl-glutamine [62–64], are stable at 37 °C and help alleviate this concern. Therefore, the use of media utilizing these stabile forms of glutamine is highly recommended. With modern embryo culture media, utilizing minimal amino acid concentrations and inclusion of dipeptide glutamine, ammonium production is minimal. Of note, while dipeptides may reduce ammonia production, they may not be as readily used by embryos as the individual component amino acids [65, 66].

12.2.2.4 Salts (Osmolality)

An important concept in regard to culture media is osmolality and its resulting effect on cell volume and ultimately embryo development. High osmolality can compromise embryo development and was one of the underlying causes of the "two-cell block" observed in early embryo culture media studies (see review [67]. Osmolality of commercial culture media vary (Table 12.1). Fortunately, active mechanisms exist in oocytes and embryos to regulate cell volume, and these mechanisms depend on media ingredients. Therefore, selection of appropriate culture media is paramount. Studies demonstrate various amino acids provide protection against high osmolality [76–78] and are thus recommended in all media to aid in this regulation [67]. Special concern exists in changing from one media to another, such as between handling media and culture media with differing ion composition, which can impact cell volume regulation. In addition, choice of culture volume can impact media osmolality due to evaporation while on heated working surfaces as well as due to air movement over culture dishes within flow hoods during dish preparation and use [79] (Fig. 12.2). Use of larger volumes of media helps combat resulting osmolality rise, and adequate oil overlay is essential, especially if using non-humidified incubators to prevent evaporation and resulting in dramatic increases in osmolality [80]. Culture in dry incubators, even under oil, can still result in evaporation and harmful osmolality increase (Fig. 12.3). This is of special concern if using an uninterrupted culture approach with a single-step medium [81]. It is useful to confirm that media osmolality is still within specific ranges following standard dish prep, especially in busy labs that may prepare numerous dishes at one time. It is also useful to confirm media osmolality following standard culture practices (drop size/time). If media osmolality is higher than acceptable ranges, dish preparation methods or culture practices can be adjusted (limit number of dishes made at one time, replenish media more frequently, increase drop size or oil overlay, etc.).

12.2.2.5 Phenol Red

Phenol red is a common media component added to media to help visually monitor pH. This may be useful to gauge media age or incubator function. Some media have chosen to exclude this indicator due to possible negative impact on embryo development. It has been suggested that possible estrogenic effects of phenol red could be detrimental. However, estrogenic activity appears to be due to the con-

Table 12.1 Various macromolecules commercially available for inclusion in IVF culture media

Macromolecule	References
Human serum albumin (HSA)	
Recombinant albumin	[68, 69]
SSS (HSA + α- & β-globulins)	[70, 71]
Dextran (DSS)	[72]
Hyaluronan	[73–75]

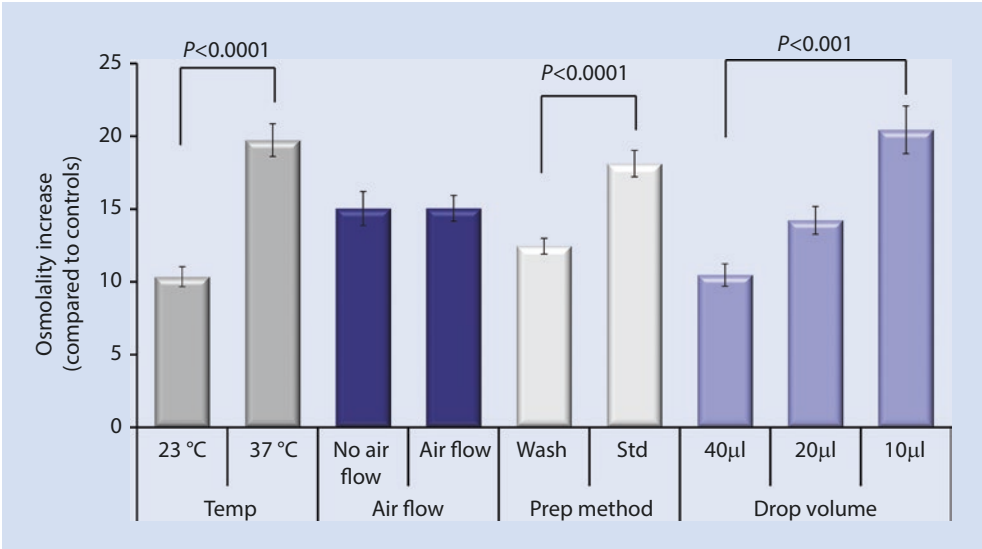

Fig. 12.2 Conditions used during preparation of microdrop dishes for embryo culture can impact evaporation and resulting media osmolality. This change in osmolality could impact subsequent embryo development

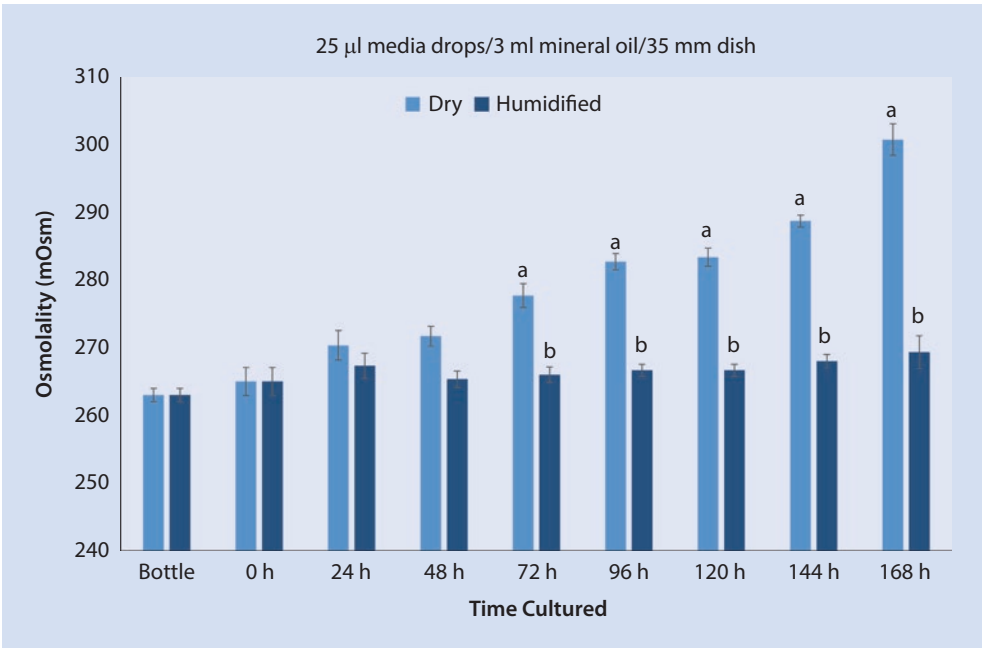

Fig. 12.3 Culture conditions can impact media evaporation during incubation, even under mineral oil. Special precautions need to be taken when culturing in non-humidified benchtop incubators, especially if using a single-step media with uninterrupted culture over 5–7 days. Different superscripts within a time point indicated a significant difference in osmolality between humidified and non-humidified culture

taminants in certain preparations not due to the phenol red itself [82]. Additionally, it has been suggested that phenol red use may lead to generation of reactive oxygen species (ROS) following light exposure [83], though this has not been shown using modern human embryo culture media. Therefore, it cannot be concluded that phenol red conveys a negative impact and its use in media may be useful.

12.2.2.6 Antibiotics

Though not required, an antibiotic of some sort should likely be included in embryo culture media. If an antibiotic is included, gentamicin, rather than penicillin/streptomycin (Pen-Strep) should be utilized. Ninety-one percent of contamination cases in IVF were Pen-Strep resistant [84], and Pen-Strep was detrimental to human and hamster embryo development [85, 86], possibly via damage to embryo chromatin, gene expression, and increased apoptosis [87]. Additionally, gentamicin is heat and pH stable, and of 70 bacterial strains in contaminated embryo media, all were sensitive to gentamicin [84].

12.2.3 Macromolecules

Macromolecules are an especially important consideration in discussions of IVF culture medium since their source, composition, and concentration are still largely decided within individual laboratories (Table 12.2). Therefore, diligence and familiarity with their components and effects are critical.

As IVF culture media advanced, the field moved toward the use of a fully defined media, and the use of maternal serum was largely abandoned. However, protein and other

Table 12.2 Osmolarity ranges of various commercial media utilized in IVF

Media	Osmolarity range (mOsM)
Irvine	
P1	282–298
ECM	282–295
Continuous Single Culture (CSCM)	260–270
Multi-blast	281–291
HTF	272–288
SAGE	
Fert Media	257–273
Cleavage Media	257–273
Blastocyst Media	257–273
1-Step	257–273
Vitrolife	
G-IVF	300 ± 5
G-1	289 ± 5
G-2	289 ± 5
GTL	270 ± 5
Life Global	
Global	260–270 (265)
Global Fert	260–270 (265)
HTF	280–292 (285)
Genea (Gems)	
Fertilization	295–305
Cleavage	285–295
Blastocyst	285–295
Origio	
Fertilization	277–293
Cleavage	272–288
Blastocyst	272–288
Cook	
Sydney IVF Fert	285–295
Sydney IVF Cleavage	285–295
Sydney IVF Blastocyst	285–295

Osmolarity can be inadvertently changed through common laboratory practices, and measures should be taken to avoid evaporation and harmful osmolarity increases

macromolecules still prove beneficial by improving embryo development, increasing cryo-tolerance, also acting as surfactants, and increasing colloidal osmotic pressure [88]. Some labs began to use commercially available serum substitute products such as Plasmanate or Plasmatein. A common protein source utilized for in vitro embryo culture is serum albumin, though lot-to-lot variation can be a concern [89], as well as the presence of various contaminants. Therefore, recombinant albumin is also used and has been found to be as effective or superior to albumin in animal models [68, 69]. Importantly, "contaminants," found in serum-derived macromolecules, may include beneficial growth factors [90]. A growing trend in human embryo culture is the use of more complex macromolecules. Inclusion of alpha and beta globulins with human serum albumin (HSA) improved human embryo development [71] and live birth outcomes [70] and may be concentration dependent [91]. Additionally, macromolecules such as hyaluronan are advantageous when included in culture media [73–75]. In vivo, embryos are surrounded by various proteins and macromolecules. Thus, they likely play a physiologic role in supporting embryo growth. Potential benefit of these macromolecules may stem from nutritional roles but may also be tied to improvement of the physical environment stemming interactions with water as suggested by Pool and Martin [71]. These authors postulated that the more pronounced interaction of water with glycoproteins, compared to albumin, might be beneficial in the in vitro environment. As a test of this, dextran, a linear polymer of glucose and a molecule known to interact strongly with water, was added to albumin as a medium supplement [72]. This combination supported embryonic developmental rates equivalent to albumin with alpha and beta globulins and produced clinical pregnancy [71, 72]. It should also be mentioned that macromolecules might be advantageous with respect to embryo transfer media. Hyaluronan has been suggested as being advantageous for transfer and embryo implantation, though conflicting data exist [92–100]. The potential for benefit is unclear as the compound cannot penetrate the zona pellucida to access the embryo and no receptors are present on the zona pellucida, though one cannot rule out direct uterine effects. Another potential rationale is that increased viscosity of transfer media may be advantageous to promote adherence of embryos to the uterine wall and can be accomplished through the use of increased protein concentrations. Another important factor to consider and monitor when utilizing macromolecules is their effect on media pH.

12.2.4 pH

While a large amount of attention in regard to IVF culture media has focused on concentrations of energy substrates or supplemental additives, the very basic principle of acid-base balance is often overlooked. Though this parameter is routinely indirectly measured during daily laboratory quality control assessments of the atmospheric CO_2 content of our incubators, it is often not appreciated that we are in fact attempting to monitor medium pH (external pH, pHe). This is a very important consideration because, though commercial

media companies now control many aspects of media production and quality, the laboratory itself directly controls this aspect of culture media.

The pHe of culture media is the measure of hydrogen ion concentration. This is set primarily as the result of the equilibrium between dissolved bicarbonate supplied in the media and CO_2 in the incubator, which dissolves in solution to yield carbonic acid. The importance of this parameter becomes more apparent when one stops to think about proper lab procedures, such as the rationale for limiting patient numbers in a single incubator, reducing incubator openings/closings, installation of inner incubator doors, minimizing dish time out of the incubator, and the use of oil overlay. All of these practices serve to limit the amount of external stress imposed upon gametes and embryos in an attempt to optimize the in vitro culture environment by stabilizing the CO_2 environment and ultimately pHe. Though mammalian embryos have various mechanisms to regulate their intracellular pH (pH_i) [101], including the Na^+/H^+ antiporter and HCO_3^-/Cl^- transporter, excursions in pHe are readily converted to variances in intracellular pH (pH_i), at least initially [102]. Additionally, denuded mature oocytes, as well as cryopreserved/thawed embryos, have a greatly reduced ability to regulate pH_i [103–106]. Ability to regulate pH_i in the oocytes is normally controlled by surrounding cumulus cells and does not become inherent to the egg until 3 h after fertilization. Similarly, following warming, pH_i regulatory capacity does not return around 6 h.

The need to establish a target pHe and adopt procedures to limit drift is clear when one considers pH controls several intracellular processes that can impact embryo development. Deviations in pH_i for only a few hours can have dramatic impacts. Raising (~7.4) or lowering (~6.8) pH_i in mouse embryos for only 3 h disrupts localization of mitochondrial and actin microfilaments compared to controls (~7.2) [107]. Minor rises in pH_i can dramatically impact embryo metabolism through the regulation of various enzymes, such as phosphofructokinase (PFK). Raising pH_i ~0.1–0.15 units for just a few hours significantly increased embryo glycolysis and lowered oxidative metabolism [50, 105], which can dramatically impact developmental competence. Considering pH_i regulatory capacity can be impaired for several hours after denuding eggs or warming embryos, the need for tight pHe control becomes more apparent. Finally, more recent evidence suggests that pH cannot only affect embryo development but resulting fetal development as well. Lowering the pHi of one-cell mouse embryos from 7.25 to 7.1 for 19 h resulted in significantly fewer blastocyst cell numbers, higher levels of apoptosis, and reduced fetal size/weight compared to controls [108].

12.2.4.1 Optimal pHe?

The ability of embryos to regulate pH_i is evidenced by various studies that show embryos can develop over a pHe range of ~7.0–7.4 without any discernible effect on pH_i or development [109, 110], while excursions of pHe outside this range have deleterious effects on embryo developmental competence [111–115]. However, just because blastocysts can be formed over these pHe ranges does not indicate that resulting embryo quality is equivalent. Drifting too far away from the pH_i of around 7.1 likely stresses the embryo, as more resources are required to maintain the proper pH_i. Conventional wisdom tells us that the pHe of culture media should be slightly higher than pH_i to help offset the acidification that occurs as a result of intracellular metabolic processes. Thus, many laboratories culture their embryos in the range of 7.2–7.4. However, considering that pH scale is logarithmic, a range of 7.2–7.4 encompasses a >60% change in the molar $[H^+]$, and a tighter range is likely prudent.

Unfortunately, there is likely no "optimum" pHe for oocytes and embryos, as this will vary from medium to medium based on specific ingredients [116, 117]. The amount of monocarboxylic acids, such as lactate and pyruvate, in culture media can lower pH_i [50, 51]. Additionally, amino acids, particularly glycine, taurine, and glutamine, act as zwitterions and help in buffering pH_i [50]. Thus, though pHe may be the same, embryos grown in different media with different amounts of monocarboxylic acids may have different pH_i. Along with potential species or strain-specific requirements associated with various animal models, this likely explains variations in the literature regarding acceptable and optimal pHe [43, 44, 118, 119]. Regardless, it would be insightful to see a properly controlled clinical trial to determine if culturing human embryos at a pH closer to 7.2 offers any benefit on embryo development, implantation, or live birth compared to culturing embryos in the same media at a pH closer to 7.3–7.35. The lack of consensus as to an "ideal" pHe at which to culture embryos is reflected in the wide ranges of acceptable pHe given by various commercial media companies (Table 12.3) [116, 117].

Furthermore, despite the growing trend, it remains unknown whether early cleavage-stage embryos prefer a slightly lower pHe than later stages of embryo development. A preliminary study using sibling oocyte splits and a single-step media adjusted pH by altering CO_2 levels suggests that perhaps a lower pHe (higher CO_2) during cleavage stages and a higher pH (lower CO_2) during later developmental stages may be advantageous [120]. Similar results have been reported for mouse embryos, though pH was adjusted in sequential media by changing bicarbonate concentrations [121]. However, it should be noted that adjusting pH by changing CO_2 may not yield the same outcomes as changing pH through adjusting bicarbonate levels (as is done with some sequential media by the commercial manufacturer) [122]. It is known that later stages of embryos with tight junctions, like the morula and blastocysts, can regulate their pH_i more rigorously than early cleavage-stage embryos and can thus tolerate a wider range of pHe [50]. Additionally, later stage embryos may have different requirements for bicarbonate or CO_2, as these serve roles in membrane-bound transporters and biosynthetic pathways. Interestingly, there are data to suggest a slightly more alkaline pHe may benefit fer-

Table 12.3 Recommended pH ranges for various commercial IVF culture media

Media	Recommended pH range
Irvine	
P1	7.27–7.32
ECM	7.2–7.25
Continuous Single Culture (CSCM)	7.25–7.40
Multi-blast	7.30–7.40
HTF	7.20–7.30
SAGE	
Fert Media	7.30 ± 0.1
Cleavage Media	7.20 ± 0.1
Blastocyst Media	7.30 ± 0.1
1-Step	7.30 ± 0.1
Vitrolife	
G-IVF	7.30 ± 0.1
G-1	7.27 ± 0.07
G-2	7.27 ± 0.07
GTL	7.30 ± 0.1
Life Global	
Global	7.2–7.4 (7.27–7.32)
Global Fert	7.2–7.4 (7.27–7.32)
HTF	7.2–7.4 (7.27–7.32)
Genea (Gems)	
Fertilization	7.30–7.50 (7.3–7.4)
Cleavage	7.20–7.50 (7.3–7.4)
Blastocyst	7.25–7.45 (7.25–7.4)
Origio	
Fertilization	7.35
Cleavage	7.2
Blastocyst	7.3
Cook	
Sydney IVF Fert	7.3–7.5
Sydney IVF Cleavage	7.3–7.5
Sydney IVF Blastocyst	7.3–7.5

CO_2 will need to be adjusted by the laboratory to obtain the recommend pH range. Some companies recommend tighter ranges than others. (Data in parentheses indicate tighter recommended ranges from the manufacturer that may not be indicated in product literature)

tilization. Dale et al. [102] found higher rates of sperm binding to empty zonae pellucidae at pHe 7.5 compared to lower pHe. This has led to the common practice of fertilizing oocytes in a slightly higher pH, culturing day 1–3 embryos in a slightly lower pH and culturing day 4–6 embryos in a slightly higher pH (high-low-high paradigm).

12.2.5 Handling Media and Buffers

Due to the crucial nature of pH in gamete and embryo physiology, the importance of IVF handling media used for manipulations outside the incubator is also an important consideration [116, 117, 123–126]. These media are formulated to maintain appropriate pHe in room atmosphere, outside the elevated CO_2 concentrations of the incubator. Though techniques such as oil overlay can combat this pH rise, dishes of bicarbonate-buffered culture media kept out of the incubator with oil for even brief periods can result in pH rising above 7.4 [124, 125, 127]. Thus, for procedures such as oocyte retrieval, sperm processing, oocyte denuding, ICSI, and embryo transfer, it is often recommended that special handling media be utilized that resists changes in pHe. In the past, handling media included phosphate-buffered saline solutions (PBS), and some laboratories continue to use this medium for oocyte retrieval. However, PBS lacks essential components, such as bicarbonate and metabolic substrates. These inadequacies, coupled with elevated levels of phosphate, may compromise gamete and embryo metabolic activity, disrupt organelle distribution, and interfere with intracellular ionic homeostasis, including intracellular pH [128–130]. Indeed, even brief exposure to PBS as a handling media has been shown to compromise hamster and rabbit embryo development [131, 132] and results in aberrant gene expression in bovine embryos when compared to other buffers [133]. Therefore, a better choice, and an approach that has benefited IVF, entails adoption of handling media that contain appropriate energy substrates, bicarbonate (though at reduced levels to help maintain pHe), and synthetic organic buffers to maintain media pH within a desired range at room atmosphere. These buffers, commonly referred to as Good's buffers, are organic compounds derived from zwitterionic amino acids that provide supplemental buffering capacity over physiologic pH range of approximately 6.1–8.3 [134–136] (Fig. 12.4a). However, different cell types display varying sensitivity to individual zwitterionic buffers [134]. Thus, determining the suitability of specific buffers for use with mammalian gametes and embryos in IVF is crucial. Two of these commonly used in commercially available handling media for ART are 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 3-(N-morpholino)-propanesulfonic acid (MOPS) and are selected based on their pKa values, an indication of their optimal buffering capacity.

a

Buffer	pH range	pKa 25 °C	pKa 37 °C
MOPS	6.5–7.9	7.20	7.02
TES	6.8–8.2	7.40	7.16
HEPES	6.8–8.2	7.48	7.31
DIPSO	7.0–8.2	7.60	7.35
MOBS	6.9–8.3	7.60	n/a
TAPSO	7.0–8.2	7.60	7.39

b

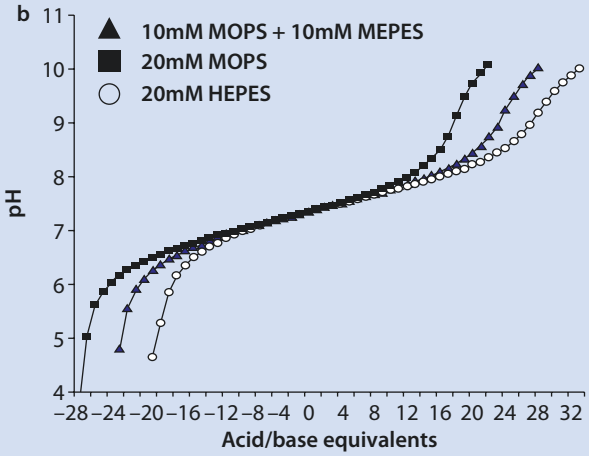

Fig. 12.4 **a** Common zwitterionic (Good's) buffers and their pKa values (optimal buffering) in relation to temperature. **b** Combining buffers allows for adjustment of pKa at specific temperatures

Historically, HEPES at ~21 mM has been a standard for IVF handling media, though commercial companies may also include MOPS or a mixture of the two compounds [124–126]. Both buffers are efficient, and laboratories using media containing these buffers yield high success rates. However, though HEPES has been widely used in IVF for years, its suitability for procedures has been questioned [137, 138]. Importantly, many of the conclusions drawn concerning toxicity of HEPES are often derived from a progenitor literature that has been cited incorrectly and the conclusions are not fully supported by the studies performed. In considering these pharmacologic effects, it is essential to note that many are largely dependent on interactions with other compounds in the media, not due to toxicity of the buffers themselves. Thus, it is important to examine the use of these buffers in the context of specific base medium. Early somatic cell studies citing HEPES toxicity stemmed from light exposure and interactions with riboflavin [139, 140]. This may have also been a factor in the observations of Butler et al. [141]. Though no data were presented, the authors commented that inclusion of HEPES in the medium resulted in increased oocyte degeneration; however, the medium contained riboflavin. Several studies actually indicate HEPES is able to support oocyte maturation [142–144], fertilization [144–148], and embryo culture [137, 144, 147, 149–152]. Those studies indicating lower fertilization rates in the presence of HEPES are likely due to the simultaneous reduction in bicarbonate concentrations [148]. Embryo development is supported in the presence of HEPES when bicarbonate is present, but not when bicarbonate is absent [151]. Furthermore, when embryos are cultured at room atmosphere and compared to controls cultured in 5% CO_2, differences in development cannot be attributed to HEPES alone. Elevated CO_2 of the laboratory incubator is utilized by embryos for various biochemical processes as a carbon source [153–155] and is likely beneficial over culture at room atmosphere. It has been demonstrated that 25 mM HEPES has no adverse effect on mouse embryo development and that there are no adverse effects of up to 50 mM HEPES when cultured with 25 mM $NaHCO_3$ in ~5% CO_2 [124, 125]. Though there may be species-specific sensitivities to HEPES, or other buffer systems, data indicate that when adequately controlling for other factors such as osmolality, bicarbonate levels, gas levels, and pH, these buffers are able to successfully support mammalian embryo development.

Temperature is another variable to be aware of regarding the use of zwitterionic buffers in IVF handling media [124–126]. Though it is known that temperature can affect pH probe readings and should be accounted for when measuring pH in this respect, it is often not appreciated that the pKa of synthetic organic buffers, as well as the actual pH of the media, changes as temperature changes [124, 125]. As an example, MOPS was presumably selected as an alternative to HEPES for IVF media not only due to toxicity concerns but also because its pKa of 7.2 is the closest of the 20 zwitterionic buffers to the pH_i of embryos of 7.12 [156]. Thus MOPS would seemingly offer the best pH buffering of available options. However, the pKa of 7.2 for MOPS is at 25 °C. Many laboratories warm their handling media to around 37 °C, a temperature at which the pKa for MOPS is actually 7.02. This is low, considering many labs set their media pH between 7.2 and 7.4. Thus, utilization of MOPS alone, or any other single buffer for that matter, may not offer ideal pH buffering of IVF.

12.3 Future Directions

12.3.1 Improved Handling Media

A novel concept to address concerns with buffer toxicity in handling media, as well as the issue of temperature and optimal pH stability, is the combination of several organic buffers into a single solution [157]. Combining buffers allows for selection of an exact pKa value at a given temperature, which may be more appropriate and offer better buffering than a single buffer (Fig. 12.4b). Additionally, superior buffering and combination of buffers allows for the use of lower individual buffer concentrations, thus lowering possible toxicity concerns and may be superior to current handling media with commonly utilized 21 mM of buffer. Indeed, this approach is a valid substitute for current mono-buffered handling media. Swain and Pool [124, 125] verified that bi- and

tri-buffered media with combinations of zwitterionic buffers support mouse embryo development. Preliminary data suggest these buffers may be beneficial for ICSI over mono-buffered media [124, 125]. With further testing and verification, additional buffers such as DIPSO or TES or their combinations with HEPES and MOPS could be explored and added to further refine media. In addition, specialized handling media for varying cell types may offer an avenue for further improvement. Just as sequential media have been developed to meet the differing needs of the embryos, formulating handling media to meet the differing needs of sperm, oocytes, and varying stages of embryos may be beneficial.

12.3.2 Improved Media Stability

12.3.2.1 Stable Additives

Supplementation of media with various additives has proven to be a major source of IVF culture improvement. Use of the dipeptide form of glutamine has proven to be beneficial due to its increased stability in culture and resulting decrease in ammonia production [62–64, 158]. A similar approach could be explored with other amino acids such as glycine [65] or other ingredients, such as pyruvate. In addition to its role as a metabolic substrate, pyruvate also acts as a powerful antioxidant [159] and can protect embryos from harmful reactive oxygen species (ROS) [160–162]. However, sodium pyruvate, the form of pyruvate added to all current embryo culture media formulations, is labile in solution [163]. By-products of pyruvate breakdown interfere with key steps in the TCA cycle, thereby compromising metabolic activity [163, 164] and are not able to scavenge ROS. Preparations of sodium pyruvate stored in media at 5 °C for 1 month may produce enough by-product to inhibit embryo metabolism [165]. A potential improvement over current use of sodium pyruvate in embryo culture media entails the use of esterified forms of pyruvate, such as ethyl- or methyl-pyruvate. These esterified forms of pyruvate have proven superior to sodium pyruvate in various experiments in somatic cells examining their protective effects against various stress paradigms [166–176]. Interestingly, in preliminary studies, both ethyl and methyl pyruvate were superior to sodium pyruvate in supporting mouse embryo development when pyruvate was the sole energy source [177] and appeared superior for embryos from mice of advanced maternal age [178]. Thus, these esterified forms of pyruvate may lend added benefit due to increased stability in solution or increased membrane permeability, factors that could have an impact on both or embryo metabolism or ability to scavenge ROS.

12.3.2.2 Progressive Media and Microfluidics

An intriguing possibility to improve upon current IVF culture media lies in its use in conjunction with another technological development known as microfluidics [179–184]. Media could be flowed over or around embryos in an occasional or continuous fashion to replenish nutrient, remove by-products, or change media composition. Through the use of automated microfluidic devices, one could potentially integrate in-line, real-time assays to monitor growth conditions. Nanosensors could theoretically detect changes in the growth environment or sense other cues from the developing cells and subsequently modify the culture environment seamlessly, without the need for removing dishes from the incubator or manually moving embryos. In essence, this would improve upon current monoculture or sequential culture systems and on practices such as splitting all cases between two media [185], by providing a customized growth environment of endless compositional possibilities suited to the specific needs of particular embryos, rather than expecting all embryos to adapt to a predetermined environment of just one or two media formulations.

12.3.2.3 Lyophilized Media/Dishes

Another approach to improve the stability of media components is through the use of lyophilized media. Lyophilized media avoids component degradation from being in aqueous solution for extended periods. This permits storage for longer periods of time, with reconstitution at the time of use. While reconstituting lyophilized media in bottles in not overly novel, one could envision having spots/drops of lyophilized media adhered to specialized culture dishes. Water could be added immediately prior to use at a time sufficient enough for equilibration, but not for extended periods where media components can degrade and harmful by-products produced. Coupled with a microfluidic system, one could envision an automated pipette system or media flow within the incubator to a discrete area of lyophilized media to rehydrate and equilibrate the media and then move the embryo to the new media. This would avoid degradation over time and would be compatible with uninterrupted culture approaches without the need to remove the dish from the incubator, thus permitting a sequential or multistep media system in an uninterrupted fashion.

Review Questions

1. There are two main approaches to embryo culture media. Regardless of the approach utilized, one energy substrate is required to permit cleavage-stage embryos to undergo the first cell division. This energy substrate is:
   (a) Glucose
   (b) Amino acids
   (c) Lactate
   (d) **Pyruvate**
   (e) Glutamine

2. The laboratory is trialing a new culture media that is presupplemented with protein. This is a departure from their normal medium, where protein is supplemented within the laboratory. Upon initial use and following overnight

equilibration in the same incubators they used with their old medium, it is noted that the color of the culture media appears more yellow than that previously observed. What would likely correct this?

(a) Raise incubator CO_2 levels
(b) **Lower incubator CO_2 levels**
(c) Raise incubator O_2 levels
(d) Lower incubator O_2 levels

3. What is the closest approximate internal pH of embryos that helps establish the recommended pH range of embryo culture media?

(a) 6.9
(b) **7.1**
(c) 7.3
(d) 7.5
(e) 7.7.

4. The best way to decrease the pH of culture is to:

(a) Increase CO_2 and increase bicarbonate concentration
(b) Increase CO_2 and decrease protein supplementation
(c) **Increase CO_2 and increase protein concentration**
(d) Decrease CO_2 and increase bicarbonate concentration

5. Perhaps one of the most researched substrates, this carbohydrate is not required for early cleavage and can inhibit early embryo development. It is used primarily by morula and blastocysts.

(a) Pyruvate
(b) Lactate
(c) Amino acids
(d) **Glucose**
(e) Glutamine

6. High levels of ammonium in culture media can impair embryo metabolism and development and possibly affect offspring. The most appropriate method to reduce ammonium production in culture media is to:

(a) Lower media pH
(b) Use a single-step medium
(c) Utilize a sequential medium
(d) Supplement with complex protein supplements
(e) **Utilize a medium containing a dipeptide**

7. This amino acid is primarily responsible for ammonium build-up in culture media.

(a) Glycine
(b) Taurine
(c) **Glutamine**
(d) Alanine
(e) Methionine

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Oocyte Preparation for IVF/ICSI

Contents

Assessment of Oocyte Quality

Basak Balaban

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Learning Objectives

- The maturity stage of the oocyte is of major importance for the successful fertilization outcome after in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI). Visualization of the morphological appearance of mature metaphase II stage (MII) oocyte may also play an important role on the clinical outcome specifically for ICSI cycles at which the cumulus-corona cells are removed before the fertilization procedure.
- The objective of this chapter is to provide an overview of the effect of morphological deviations of MII oocytes on the clinical success in assisted reproduction.

Key Points

The deviations that should be examined with high priority are in the following order:

- Oocytes that are large in size (giant oocytes) and oocytes that have a large first polar body should not be used for insemination because of the high risk of chromosomal abnormalities. If the patient has only such oocytes, preimplantation genetic screening of the derived embryo can be recommended.
- Oocytes should be observed for the presence of smooth endoplasmic reticulum cluster/s within the cytoplasm. The patient should be informed that embryos derived from such oocytes may have significantly reduced rates of healthy offspring.
- Oocytes should be observed for the presence of vacuole/s within the cytoplasm. Patients should be informed that MII oocytes with vacuole(s) ≥14 μm have a significantly lower chance of getting fertilized when compared with oocytes with a normal morphological appearance.
- Oocytes should be observed for the presence of organelle clustering/centrally located condensed granulation within the cytoplasm. The patient should be informed that embryos derived from such oocytes may have a higher risk of chromosomal abnormalities.
- Oocytes defined with other cytoplasmic deviations such as refractile bodies/cytoplasmic inclusions or with dark cytoplasm/dark cytoplasm-granular cytoplasm/dark cytoplasm with slight granulation/dark granular appearance of the cytoplasm/diffused cytoplasmic granularity should be documented.
- Ovoid oocytes with ovoid zona and normally shaped oolemma or ovoid zona and ovoid oolemma should be observed as the blastocyst formation rate of embryos derived from such oocytes may be detrimentally affected and delayed.
- Oocytes with extremely large perivitelline space (PVS) may result in reduced fertilization rates and higher degeneration rates following ICSI.
- Dysmorphic zona pellucida, discoloration of the oocyte, first polar body morphology, and debris in PVS should be documented.

13.1 Assessment of Oocyte Quality by Morphology

Assessment of oocyte morphology and determination of its correlation with quality/viability and the clinical outcome are difficult tasks, since the underlying mechanisms that change the appearance are multifactorial and complex. Optimal oocyte morphology is defined as an oocyte with a spherical structure enclosed by a uniform zona pellucida, with a uniform translucent cytoplasm free of inclusions and a size-appropriate polar body [1, 2]. However, metaphase II stage (MII) oocytes retrieved from patients after ovarian stimulation are known to show significant morphological variations that may affect the developmental competence and implantation potential of the derived embryo.

More than half of the oocytes collected can contain at least one morphological abnormality, and this may be correlated with the asynchrony between nuclear and cytoplasmic maturations of the MII oocyte, which plays an important role on its viability and the clinical outcome. Morphological variations of the oocytes may also result from other intrinsic factors such as age and genetic defects or extrinsic factors such as stimulation protocols, culture conditions, and nutrition.

Conflicting results are published in the literature regarding the effect of morphological variations of the oocyte on embryo development and implantation.

This chapter reviews the correlation of morphological abnormalities of the MII oocyte and the clinical outcome and the effect on genetic disorders, discusses the predictive value of specific abnormalities, and examines whether any of these parameters can be utilized in all the scoring systems applied in in vitro fertilization (IVF) laboratories.

Morphological abnormalities of the oocyte will be observed under two different subgroups: extracytoplasmic abnormalities and cytoplasmic abnormalities [1–4].

13.2 Morphological Deviations of Mature Metaphase II Stage Human Oocytes

13.2.1 Cytoplasmic Abnormalities

Cytoplasmic abnormalities of MII oocytes include different types and degrees of cytoplasmic granulations (slightly diffused or excessive whole/centrally located granulation) and appearance of refractile bodies, smooth endoplasmic reticulum clusters (sERCs), or vacuolization in the ooplasm. Detection of cytoplasmic variations was first termed as cytoplasmic dysmorphism by Van Blerkom and Henry in 1992 [5] and since then used as a selection method to assess the viability and implantation potential of the derived embryos.

13.2.1.1 Morphological Appearance of the Cytoplasm

Although the appearance of cytoplasm was considered as a potential predictive factor for the success of clinical outcome, different definitions and groupings of multiple morphologi-

cal features in various published studies make the comparative analysis difficult. The terminologies used in the literature are dark cytoplasm [6–8], dark cytoplasm-granular cytoplasm [9], dark cytoplasm with slight granulation [10], dark granular appearance of the cytoplasm [11], and diffused cytoplasmic granularity [12]. High subjectivity of the definition of these types of granulations in various laboratories provides limited predictive value for the clinical outcome. Despite the fact that the majority of the clinical trials examining the effect of dark granular cytoplasm as an individual feature showed no detrimental effect on the viability of the derived embryo [6, 9–11] and even may be associated with higher fertilization when compared with the group of oocytes with total absence of granularity [13], controversial results were also published by other authors [7, 8, 12]. Variations published in these articles may be correlated with the subjectivity of the definition, as diffused slight cytoplasmic granularity, so-called also as dark cytoplasm, can be ill-defined and could differ by different modulations of the optical path in phase-contrast microscopies in various laboratories. Homogeneity of the cytoplasm is expected; however, biological significance of non-homogeneity is unknown, and based on current evidence, it may only represent variability between oocytes rather than a dysmorphism of developmental significance [1].

Centrally Located Granulation of the Cytoplasm

Condensed granulation that is centrally located within the cytoplasm with a clear border is unlike various degrees of diffused granulation described above, as it is easily distinguishable with a significant darker appearance than normal cytoplasm, which could be clearly visible by any modulation type of the optical path in different phase-contrast microscopies. It had first been defined by Serhal et al. [14] with a detrimental effect on the outcome of intracytoplasmic sperm injection and named as centrally located granular cytoplasm (CLCG) by Kahraman et al. [15] later. It was indicated that CLCG is a rare morphological feature of the oocyte that is diagnosed as a large, dark, spongy granular area in the cytoplasm, and the severity is based on both the diameter of granular area and the depth of the lesion.

Even though fertilization rate and embryo quality were not affected in the study by Kahraman et al., poor ongoing pregnancy chance correlated with high aneuploidy (52.3%), and abortion rates (54.5%) were obtained by the transfer of embryos derived from oocytes with severe CLCG. The study by Meriano et al. [16] defined CLCG oocytes as organelle clustering and found out that this certain abnormality is the only repetitive dysmorphism in consecutive cycles and is a negative predictor of pregnancy and implantation rates in intracytoplasmic injection cycles. Even though more research is needed to define the subcellular and molecular mechanisms of organelle clustering, hypoxia of the follicle was shown to be correlated to oocytes of such poor developmental competence [17]. It has also been demonstrated that MII oocytes that exhibited severe cytoplasmic disorganization had a lower intracytoplasmic pH and ATP content as well as increased incidence of aneuploidy and chromosomal scattering [18, 19], which was later confirmed by Kahraman et al. as well [15]. A more recent study by Yakin et al. [20] had also shown that the embryos that were derived from oocytes with severe cytoplasmic abnormalities where CLCG oocytes were included had a higher rate of aneuploidy rate (60.0%) when compared with group of embryos derived from oocytes with a normal morphological appearance (41.9%). However, the results were not statistically different most likely based on insufficient number of embryos included in the comparison. The same group demonstrated that cryopreserved day 3 cleavage stage embryos derived from oocytes with severe cytoplasmic abnormalities, where CLCG oocytes consisted the majority of the experimental group, had a significantly lower cryosurvival rate, and even if they survived, they had lower rates of blastocyst formation, and none of the blastocysts obtained were of good quality, and none were able to successfully complete the hatching process [10]. Based on the evidence proof, it is important to inform patients about such morphological defect of the MII oocyte and the reduced implantation outcome, as well as increased risk of aneuploidy with the resultant embryos [1].

Refractile Bodies

Refractile bodies are so called as cytoplasmic inclusions, which can be dark incorporations, fragments, spots, and dense granules: lipid droplets and lipofuscin. Transmission electron microscopy studies and Schmorl staining had shown that refractile bodies over 5 μm showed the conventional morphology of lipofuscin inclusions consisted of a mixture of lipids and dense granule materials [21]. Lipofuscin bodies in human oocytes can be detected throughout meiotic maturation (GV, germinal vesicle; MI, metaphase I; and MII, metaphase II stage), which is different from other cytoplasmic abnormalities in humans, such as smooth endoplasmic reticulum clusters, which appear only in mature MII oocytes.

The average diameter of a recognizable refractile body under bright-field microscopy is approximately 10 μm [22]. As in the majority of the published articles, cytoplasmic abnormalities are examined jointly, whereas data on the individual predictive value of refractile bodies on clinical outcome are limited. According to some investigators, cytoplasmic inclusions did not appear to affect fertilization, embryo quality, and implantation rates [5, 8]. A more recent study by Rienzi et al. [12] has also shown that refractile bodies do not detrimentally affect fertilization and normal pronuclear morphology rates. In contrast, Xia [23] and Otsuki et al. [21] reported a decreased fertilization rate and embryo development. It is most likely that controversial results might be correlated to the factors that are still unknown, and one possible confounding factor could be the differing diameters of refractile bodies. Only one study in the literature had examined precisely the relationship between the sizes of the refractile bodies and developmental competence of oocytes and had found that lipofuscin inclusions were associated with reduced fertilization and unfavorable blastocyst development only when their diameter is over 5 μm. This study

had also shown that the size of refractile bodies is not correlated with the age of women, or different stimulation protocols, and the embryo developmental outcome was not significantly affected by stimulation regimes [21]. According to the outcome found by Otsuki et al. [21], the aging of oocytes during inactive phases of oogenesis may not be involved with lipofuscinogenesis; instead, the accumulation of lipofuscin may occur during the growth phase of the oocytes when dominant follicles are being recruited into the preovulatory pathway. The occurrence of large lipofuscin bodies in normal aging may also be related to conditions of the developing ovarian follicles, such as perifollicular blood circulation and follicular fluid composition. Other explanations can be related to oxidative stress [24], proteolytic degradation [25], or lipid metabolism as a source of energy supply [26]. Further research will be needed to investigate whether any of these possibilities are involved in refractile bodies mainly, which are mainly correlated with lipofuscinogenesis in human oocytes.

Vacuoles

Vacuoles are membrane-bound cytoplasmic inclusions filled with fluid that is virtually identical with perivitelline space liquid [27]. Their sizes and numbers may vary, and it is assumed that vacuoles arise either spontaneously [27] or by fusion of pre-existing vesicles derived from smooth endoplasmic reticulum and/or Golgi apparatus [28].

The incidence of vacuoles in MII oocytes varies from 3.1% [11] to 12.4% [29]. However, multiple vacuolization is a less likely seen phenomenon with approximately 1%–1.5% [6, 7, 30]. The study by De Sutter et al. [6] reported a severely reduced fertilization rate in vacuolated oocytes (40%) compared with oocytes without any vacuoles. Ebner et al. [30] had also reported a significantly decreased fertilization rate for oocytes containing single vacuoles (51.6%) and multiple vacuoles (43.8%) compared to oocytes without vacuoles (65.3%). A more recent study by Rienzi et al. [12] demonstrated a significantly reduced fertilization rate for vacuolated oocytes; however, pronuclear morphology and embryo quality were not detrimentally affected. Only one study [30] had a subgroup analysis examining the effect of the size of the vacuoles on fertilization rates and in fact found out that the group of oocytes that fertilized normally in between vacuolated oocytes contained vacuoles with less than 9.8 ± 3.7 μm diameter, which was significantly smaller than the diameter of vacuoles that the unfertilized oocytes contained (17.6 ± 9.0 μm). A cutoff value of 14 μm for vacuole diameter was noted, above which fertilization did not occur. This study had shown that a larger vacuole or multiple vacuoles may cause a much more detrimental effect to the oocyte than a small vacuole, since a larger portion of the cytoskeleton (e.g., microtubules) cannot function as it is supposed to. The study by Van Blerkom et al. [27] also suggested that large vacuoles might displace the metaphase II stage spindle from its polar position, which in turn can result in fertilization failure, cleavage abnormalities, and/or an abnormal cytokinesis pattern. Even though the presence of few small vacuoles (5–10 μm in diameter) that are fluid filled but transparent is unlikely to be of biological consequence, observation of large vacuoles bigger than 14 μm should be noted [1]. Besides the deficiency on fertilization rates, it has also been shown that blastocyst formation, good quality, and hatching blastocyst rates can significantly decrease after intracytoplasmic sperm injection of vacuolated oocytes. The percentage of aneuploid embryos can also be affected by the utilization of vacuolated oocytes (41.9%) when compared with embryos that are derived from oocytes with a normal morphology (60.0%) [20]. Vacuolization in MII oocytes can also decrease cryosurvival rates and subsequent embryonic development of the derived cryopreserved embryos [10].

Smooth Endoplasmic Reticulum Clusters (sERCs)

The presence of sERCs in the cytoplasm of MII oocytes is one of the most important cytoplasmic defects that need to be carefully examined. Correlation between the presence of sERCs in MII oocytes and the clinical outcome was first published by Otsuki et al. [31]. Otsuki reported that in approximately 10% of the cycles, cytoplasmic localization of translucent vacuoles similarly sized as pronuclei exists at the metaphase II stage of human oocytes after the denuding procedure for intracytoplasmic sperm injection. This incidence was 5% in the study of Mateizel et al. [32], whereas it was 7% in the study of Braga et al. [33]. However, it is most likely that the number of oocytes with sERCs can be underestimated, as it has been shown by transmission electron microscopic analysis that there are at least three forms of sERCs: large (18 μm); medium (10–17 μm), which can be classified by light microscopy; and small (2–9 μm), which are not visible under the conditions used in clinical embryology laboratories for examination. sERCs can easily be distinguished from fluid-filled vacuoles since they are not separated from the rest of the ooplasmic volume by a membrane and seen as translucent vacuoles. Even though the mechanism responsible for sERCs is still unknown, there are some human and animal studies assuming that it could be correlated to some functional and structural alterations of ER during oocyte maturation such as an increase in the sensitivity of the IP3 receptor for Ca^{2+} [34]; increased storage of Ca^{2+}, which is released during oscillation [35]; changes in the structure from a sheet-like form to a spherical form in starfish oocytes [36]; and distribution of the ER in mouse oocytes [37]. In the human oocytes, the localization of mobilizable Ca^{2+} was detected in the small vesicles beneath the plasma membrane of sER. Otsuki et al. [31] compared the clinical outcome of patients with oocytes containing sERCs and patients with retrieved oocytes without sERCs and examined whether any confounding parameters such as stimulation methods and hormonal levels can affect the outcome. Fertilization rate and embryo quality were not detrimentally affected; however, significantly lower clinical pregnancy and implantation rate and significantly higher biochemical pregnancy were observed for sERC-positive cycles. Due to the limited number of sam-

ples tested, no significant differences were found between the study groups when stimulation protocols were compared. However, the number of sERC-positive oocytes obtained by the short protocol was about three times larger than that by the long protocol. Serum estradiol levels on the day of hCG administration were significantly higher in sERC-positive cycles. This study had clearly shown that the viability of an embryo is significantly reduced with the presence of sERCs. Even though the embryo is derived from an oocyte without any clusters, its implantation potential is detrimentally affected if the oocyte is from the cohort of oocytes where at least one oocyte is with clusters. Besides the viability of the derived embryos, the most important issue with the presence of sERCs had been the neonatal safety based on evidence proof in the literature. In the study by Otsuki [31], only one pregnancy derived from gametes with an sER defect has been reported, in which the baby was diagnosed with Beckwith-Wiedemann syndrome.

The study by Ebner et al. [38] had shown that the occurrence of sERCs is significantly related to longer duration and higher dosage of the stimulation. Fertilization and blastocyst formation rate were significantly lower for oocytes with sERCs when compared with oocytes without sERCs. Take-home baby rate was significantly lower in the group with sERCs, and miscarriage rate was significantly higher in the same group of patients. Pregnancies in women with affected gametes had a significantly higher incidence of obstetric problems. Birth weight of babies born in the group with sERCs was significantly lower, and there were two unexplained neonatal deaths reported in the group with affected gametes, whereas there were no deaths reported in the group without sERCs. The malformation rate was similar in both groups, with one case of diaphragmatic hernia reported in the group with sERCs. Similar findings reported by Ebner [38] and Otsuki et al. [31] support the idea that this phenomenon is the manifestation of an intrinsic oocyte defect caused by a suboptimal ovarian stimulation [27] and perhaps as a result of overstimulation. Following other studies reporting multiple malformations [39] and ventricular septal defects [39] after the transfer of embryos originated from oocytes with sERCs, it was strongly recommended that oocytes with this feature should not be inseminated and even the sibling oocytes should be carefully examined [40]. However, a very recent study [32] in which 394 sERC-positive cycles and 6845 sERC-negative cycles were retrospectively analyzed should lead the investigators to further research as this study reported that there was no difference in the rate of major malformations between sERC-positive cycles (5.3%) and sERC-negative cycles (82.1%). In addition, no major malformations were reported from the live born definitely originating from embryos originated from oocytes with sERCs positive. Three newborns, from single-embryo transfer with frozen-thawed embryos originating from sERC-positive oocytes, were delivered and presented no major malformations. Taking into account these recent controversial data on neonatal outcome, the fate of babies born from oocytes with this feature should be very carefully evaluated and reported.

13.2.2 Extracytoplasmic Abnormalities

A variety of extracytoplasmic anomalies exist, which in part negatively influence fertilization (consistency and thickness of the zona, polar body (Pb)1 decay, and debris within the PVS), blastulation (thickness of zona, Pb1 fragmentation), and pregnancy (thickness of zona, Pb1 morphology, debris in PVS). Characteristics of the zona pellucida and the PVS are most probably associated with the health of the developing follicle, e.g., its vascularization and oxygen content. Any disturbance during growth might severely alter oocyte morphology resulting in a pool of gametes with different prognoses.

13.2.2.1 Dysmorphic Zona Pellucida

As a result of the mutual dependence between somatic cells (e.g., cumulus cells) and the egg, it is likely that any disturbance negatively affecting the follicle will have a comparable impact on the oocyte itself. Among the conceivable changes in oocyte performance, it is possible that the secretion/patterning of the zona pellucida (ZP) from the secondary follicle onward could be altered or interrupted [41, 42]. This could result either in dysmorphism that can be seen under a light microscope or more subtle changes of the three-dimensional structure of the ZP.

Definitely the most severe form of impaired growth of the zona pellucida is its complete absence. Normally, up to four zona proteins [43, 44] build up the three-dimensional matrix of the outer protective shell. Filaments are constructed of repeating zona protein (ZP) 2 and 3 units, which are cross-linked by ZP 1 [45], thus contributing to the structural integrity of the ZP. Experiments in mice lacking the ZP1 gene showed that secreting only ZP2 and ZP3 results in a thinner, more loosely organized zona pellucida [46]. On the other hand, disruption of ZP 2 and 3 led to complete absence of the acellular coat resulting in infertility [47].

In humans, a defect in gene expression was shown to cause a failure in glycoprotein matrix, even though the ovum itself showed intact corona cells [48]. In such rare cases, the ova fail to fertilize in conventional IVF. In ICSI, there is a considerable risk of exposing the gametes to mechanical stress during the denudation process. Stanger et al. and others [48, 49] showed that in patients with zona-free eggs, pregnancies can be achieved by simply leaving the coronal cell layer attached, as it acts as a supporting structure keeping the oocyte in shape during injection.

From conventional IVF, it is known that thicker zonae (e.g., >20 μm) are associated with lower fertilization rates [50]. This has been linked to patient and stimulation parameters [51]. In ICSI, however, a thicker zona interferes with neither subsequent fertilization nor implantation since assisted hatching can be applied.

Recently, it has been published that the multilaminar structure of the ZP can also be analyzed quantitatively using polarized light microscopy [52]. Though variation exists in the thickness of zona layers around individual eggs and between members of a cohort, it is evident that the inner zona layer is the most dominant part of the zona [42, 52]. It

has been reported that the birefringence of the inner zona is directly proportional to its thickness [42, 53, 54].

Shen et al. [42] found an almost 30% higher mean light retardance in conception cycles compared to non-conception cycles, indicating that stimulated cycles may yield oocytes of affected quality. There are two retrospective studies that have suggested a relationship between ZP birefringence (inner layer) and preimplantation development. Montag et al. [54] noted a higher rate of good-quality embryos on day 3 (but not on day 2) in an oocyte group with high zona birefringence (41.7%) as compared to a cohort with low birefringence (24.4%). Others [53] observed a difference in progression to the blastocyst stage. The lower the measured retardance, the lower the blastocyst formation.

13.2.2.2 Discoloration

Irrespective of the actual thickness of the ZP, ovarian stimulation sometimes generates gametes showing a zona pellucida that appears dark or brownish under a light microscope. Mostly, the egg itself is affected as well. In the literature, it is reported that the presence of discolored ZP is a common phenomenon, e.g., 9.5–25.7% [6, 9, 29, 55].

It has to be kept in mind that it is not completely clear that dark or brown zonae/oocytes occur for the same reasons. Recently, these oocytes were termed "brown eggs" since they were found to be dark with a thick ZP, a rather small perivitelline space (sometimes filled with debris), and granular cytoplasm [11]. Esfandiari and coworkers [11] prospectively compared the outcome of brown gametes with that of gametes of normal appearance. Although the zona in discolored eggs were thicker than that of control gametes, in conventional IVF, the fertilization rate was similar. The same was true for the fertilization after ICSI, embryo quality, implantation rate, and clinical pregnancy rate. However, it has to be mentioned that because of the thick zonae, brown oocytes were subjected to laser-assisted hatching significantly more often than the control group.

13.2.2.3 Shape Anomalies

Even if the thickness or color of the zona pellucida is inconspicuous, it is not automatic that the shape of the gametes is spherical. Indeed, there is evidence that extremely ovoid eggs exist [56]. Such gametes have been shown to be fertilizable and may lead to the birth of a healthy baby. However, a major problem with these reports is that the degree of the shape anomaly was not quantified, and rather more imprecise descriptions have been given (e.g., cucumber shaped).

Recently, our group successfully measured ovoid oocytes [57] and calculated a roundness index (RI, length divided by width). Actually, two indices were determined to assess whether the whole oocyte was affected (showing an ovoid ooplasm and zona) or only the zona pellucida was of ovoid shape (with the ooplasm being perfectly round). Special care was taken to detect splitting of the innermost zona layer that might keep ooplasm in round shape (while zona is ovoid). The latter dysmorphism was shown to be associated with implantation failure [42].

The degree of shape anomaly was correlated to neither fertilization nor embryo quality [58], which is in line with previous data [56]. Interestingly, two types of cleavage patterns were observed on day 2. Either ovoid gametes cleaved normally like a tetrahedron (a crosswise arrangement of four cells with three blastomeres lying side by side) or, if the ovoid zona failed to exert its shaping function, resulted in a rather flat array of four blastomeres. Since the abnormal pattern reduces the number of cell-to-cell contact points from 6 to 5 or 4, compaction and blastulation of the corresponding embryos may be delayed [57, 59].

Two possible mechanisms may account for the occurrence of ovoid oocytes. First, mechanical stress during oocyte puncture and/or denudation processes could deform the egg. This unwanted occurrence would create ovoid gametes with both ooplasm and zona being affected. In these artificially damaged gametes, a tendency toward recovery within a day has been suggested [57]. Thus, for the vast majority of ovoid ova, it can be assumed that the deformation is a preexisting anomaly generated during maturation within the follicle.

13.2.2.4 Perivitelline Space

The size of the perivitelline space (PVS) is closely related to the maturational phase of the oocyte. While at germinal vesicle stage (prophase I) expansion of PVS is minimal, it begins to increase after the resumption of meiosis. In detail, at metaphase I, PVS can clearly be detected, and after completion of maturation (metaphase II stage), its full size is reached.

Several authors have noted that up to 50% of all ova show a large PVS [12, 23]. In oocytes with a larger PVS, a lower fertilization rate was observed (67%) as compared to gametes with a normally sized (85%) gap [23]. This is more or less in line with the results of an Italian group [12], which showed that a large PVS is detrimental to fertilization and zygote morphology. Interestingly, patient parameters such as female age and indication did not seem to influence PVS performance [23], but the ratio of estradiol to testosterone (and to progesterone) did [58].

Data from in vitro and in vivo matured oocytes indicate that a large PVS may be ascribed to over-mature eggs [60, 61]. In other words, such eggs have shrunk in relation to the zona pellucida presenting a large gap between them. A large PVS would also occur if a larger portion of cytoplasm is extruded together with the haploid chromosomal set during first polar body formation. This would result in a large first polar body and a large PVS.

13.2.2.5 First Polar Body Morphology

For a long time, it was thought that first polar body (Pb1) extrusion marks the completion of nuclear maturation ending in a metaphase II stage oocyte. Recently, using polarized light microscopy, it has been demonstrated that some oocytes showing a Pb1 were actually in telophase I and not in metaphase II stage [62, 64]. Otsuki et al. [65] found a chromosome aggregation phase, which occurred not only from germinal vesicle breakdown to metaphase I but also from

telophase I to metaphase II stage. In case that ICSI is performed, although chromosomes are unaligned, it may result in failed fertilization or three pronuclear zygotes due to abnormal chromosomal segregation.

The impact of 1Pb morphology on outcome is still discussed controversially. Though some 1Pbs in humans remain intact for more than 20 h after ovulation, they generally have a shorter lifetime [66]. Taking this time dependency into consideration, it might be hypothesized that 1Pb morphology provides adequate information on the actual postovulatory age of the corresponding egg [61].

Ebner et al. [55] tried to focus exclusively on the status of the Pb1. Ova showing an intact Pb1 gave rise to higher rates of implantation and pregnancy [67] probably due to an increase in blastocyst formation [68]. However, these data are still a matter of debate [12, 55, 63, 68–71].

Apparently, the benefit of selecting oocytes according to the morphology of the Pb1 is somewhat reduced with increasing time span between ovulation induction and ICSI, since studies with different schedules could not find a relationship between constitution of the Pb1 and subsequent ICSI outcome [63, 70, 71]. In these data sets, the percentage of oocytes with fragmented polar bodies was higher [23, 63, 70, 71] than that reported in the work of Ebner et al. [55] in which polar bodies were scored 2 h after collection. This is in line with the finding that of all intact Pb1, 13% were already fragmented at a second inspection 3 hours later [71].

For practical reasons and in order to minimize the risk of ovarian aging in vitro [33], ICSI should be finished within 42 h post ICSI [72].

Data from Hungary [70] suggest that a large Pb1 is the worst case. When large Pb1s were extruded, embryos with multinucleated blastomeres were significantly ($P < 0.001$) more frequent (26.7%) than in all other Pb1 classes (ca. 8%). It has been postulated that the extrusion of an abnormally large Pb1 is due to dislocation of the meiotic spindle [73]. This would in part explain the observed impact on fertilization and embryo development [12, 55, 63].

13.2.2.6 Debris in the Perivitelline Space

Sometimes it is difficult to distinguish between heavily fragmented Pb1s and debris within the PVS. Two hypotheses have been proposed in order to explain the origin of the latter dysmorphism. One hypothesis is derived from ultrastructural data indicating the presence of an extracellular matrix comprised of granules and filaments in the space between oolemma and ZP since the matrix is identical to that found between cumulus cells and the corona radiata [74, 75].

The second hypothesis is based on the existence of coronal cell processes passing the zona pellucida and reaching the egg early in maturation. It is suggested that after withdrawal of these processes, some remnants remain within the PVS [76].

The findings of Hassan-Ali et al. [77] support the latter theory since they found a close relationship between the frequency of PVS granularity and maturation. In detail, they never detected debris in prophase I eggs but found debris in 4% of the metaphase I and in 34.3% of metaphase II stage gametes. They were also able to show that the presence of PVS granules was gonadotropin dose-dependent. If less than 30 ampoules were used to stimulate the patient, 17.4% of the eggs were positive for this anomaly compared to 45.4% in high-dose patients (>45 ampoules). Fertilization rate, cleavage rate, and embryo quality were found to be unaffected by the presence of coarse granules in the PVS [77, 78]; however, rates of implantation and pregnancy seem to be affected [78], since transfer of embryos derived from PVS granule-free oocytes increased implantation rate by 5% and pregnancy rate by 21%.

13.3 Conclusions

The final endpoint for evaluating morphological abnormalities of MII oocytes is to be able to correlate it with oocyte quality and viability and in correlation increase the overall efficiency of human-assisted reproduction in terms of clinical success and safety of offspring. High heterogeneity of the published material and subjectivity of the evaluation of morphological deviations of MII oocytes may only provide limited take-home messages on the predictive value of outcome parameters for successful results. It is of major importance to use the evidence-based definitions and visualizations (e.g., normal mature oocyte, extracytoplasmic deviations like zona pellucida, shape, perivitelline space and first polar body differentiations, dense cytoplasmic granularities, refractile bodies, vacuolization, and presence of sERCs for morphological deviations) to minimize the subjectivity [79]. A recent meta-analysis [80] examining the clinical results of 40 relevant articles on previously described extracytoplasmic and cytoplasmic abnormalities showed that there was no clear tendency to a general increase in predictive value of morphological features and that these contradicting evidence proof underline the importance of more intensive and coordinated research that would lead to more objective criteria with better predictive value to determine the viability of derived embryos. Rapidly developing continuous research on new biomarkers of oocyte quality may perhaps be utilized in addition or as an alternative to morphological assessment in the future; however, current limited predictive value of morphology should not be underestimated considering that they still remain to be the sole method of choice for selection until a more effective technology can substitute its utilization in routine practice in various clinical in vitro fertilization laboratories worldwide. Beyond the predictive value of oocyte morphology, it must not be forgotten that information linking dysmorphism with genetic disorders is of great value and scarce as these disorders are directly correlated with the health of the offspring in ART applications. According to a common hypothesis [5], the vast majority of extracytoplasmic anomalies occur late in maturation since they are associated with fertilization and developmental failure rather than with aneuploidy (e.g., giant eggs). However, evidence-based data clearly demonstrate that some specific severe cytoplas-

mic defects are correlated with chromosomal aneuploidy and genetic disorders as described above. It is obvious that some anomalies, e.g., so-called giant eggs [81, 82] with an almost double-sized diameter, show a diploid chromosomal set, which contributes to digynic triploidy. Other authors [83] analyzed embryos genetically according to their polar body classes. No correlation was observed between polar body shape and genetic constitution; however, the only polar body group bearing a theoretical risk of chromosomal disorder, considering the larger volume of ooplasm in large polar bodies, was not analyzed.

Review Questions

1. How can the normal morphology be defined for mature MII human oocytes?
2. Which type of morphological deviations can a mature MII oocyte have?
3. What are the cytoplasmic abnormalities a MII oocyte may consist of?
4. Which type of cytoplasmic abnormalities is shown to affect the clinical outcome?
5. What are the extracytoplasmic abnormalities of the MII oocyte defined in the literature?
6. Can any type of extracytoplasmic abnormality affect the clinical outcome?

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Oocyte Denuding

Laura Francesca Rienzi, Roberta Maggiulli, and Filippo Maria Ubaldi

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Learning Objectives

- Indication to oocyte denudation
- Laboratory routine procedures to perform oocyte denudation
- Noninvasive assessment of oocyte quality biomarkers
- Expedients to minimize any potential damage to oocyte viability
- Future directions and improvements about oocyte denuding

14.1 Overview

Mammalian folliculogenesis is a complex, fine-regulated process during which oocytes complete their growth and meiotic maturation. Throughout the preovulatory growth, within the follicle, two different cell layers surround the oocyte, *granulosa cells (GC) and thecal cells*, which support oocyte nourishment and maturation, providing essential metabolites, hormones, and growth factors [1–4].

Whereas a passive role of the oocytes in folliculogenesis was previously assumed, at the beginning of the 2000s, it became undeniable as an oocentric perspective of folliculogenesis that highlights the central role of the oocyte itself in directing its own development, as well as follicle differentiation [5–9]. In this regard, the oocyte is deemed capable of modulating the follicular environment in order to guarantee to itself a correct preantral, antral, and preovulatory development.

During follicular antrum formation, granulosa cells differentiate in two different specialized subpopulations: the cumulus oophorus (CC), whose innermost layer, immediately adjacent to the zona pellucida, is called corona radiata, and the parietal granulosa cells. Cells of the corona radiata extend their cytoplasm toward the oocyte through the zona pellucida, creating gap junctions with oocyte microvilli. Communications (either paracrine interaction or gap junction) occur between the oocyte and the cumulus–corona cells, allowing oocyte nutrition and maturation from the diplotene to the MII stage [10, 11]. During ovulation and resumption of meiosis (induced by the preovulatory peak of LH in natural cycles), a definitive retraction of corona cell process and changes in the organization of corona radiata are observed. In parallel, the LH surge stimulates a considerable active secretion of hyaluronic acid, causing further expansion of cumulus corona mass that appears finally as an expanded and mucified layer. This organized structure is referred to as cumulus–oocyte complex (COC). Cumulus expansion is essential for ovulation and fertilization as well as capacitation and selection of sperm.

An unexpanded cumulus with multilayers of compact GCs is a typical feature of immature COCs (for instance, those that are retrieved from small follicles during in vitro maturation cycles).

While in natural spontaneous cycles, oocyte nuclear maturation runs parallel to an increased FSH-dependent expansion and mucification of the cumulus layer [12], in stimulated cycles, an asynchrony of these two processes may be observed [13]. This may be caused by a different sensitivity of the oocyte and the cumulus–corona mass to the stimulants [13, 14].

Besides their well-established role in supporting oocyte maturation, cumulus–corona cells might also be involved later on in embryonic development. These cells are supposed to be involved in embryonic metabolism, either stimulating gene expression [15] or reducing oxidative stress [16].

During assisted reproductive treatments, oocytes are removed from the follicular microenvironment and exposed for a variable amount of time to artificial culture conditions without the protection of cumulus layers and prior activation induced by the sperm entry at fertilization. The effect of this is unknown and still unexplored.

14.2 Ovum Pickup and Cumulus–Corona–Oocyte Complex Harvesting

Once maturity of the follicles is accomplished, human chorionic gonadotropin administration is used to trigger ovulation, and oocyte pickup is performed approximately 36 h later, by ultrasound-guided transvaginal aspiration.

The technical details of oocyte retrieval can differ between centers, but basically the harvesting is carried out using an aspiration unit composed of a vacuum pump, a collection tube connected to a 17-gauge needle, and a collecting test tube. Today, several sophisticated vacuum pumps with adjustable aspiration pressures are available commercially.

During the collection, a maximum pressure of about 120 mmHg is recommended to avoid the risk to damage the oocytes, such as stripping of the cumulus cells or fracturing the zona pellucida [17].

Once the ovaries are visualized, ovarian follicles are aspirated in a systematic way. Immediately after the collection, the adjoining laboratory examines the follicular fluid. COCs are identified in sterile plastic dishes, rinsed, transferred in pre-equilibrated IVF medium tube, and incubated at 37 °C in an adequate atmosphere.

During the entire procedure, efforts should be made to minimize the oocyte exposure to even transient temperature and pH fluctuations that may have a detrimental effect on oocyte competence to develop. Redding and colleagues have investigated the variation in temperature, pH, and dissolved oxygen levels associated with oocyte retrieval, in a bovine model [18]. The authors reported a significant drop (7.7 ± 1.3 °C) in temperature, a pH increase of 0.04 ± 0.01, and substantial rises in the dissolved oxygen content of follicular fluid during follicular aspiration. Therefore, in order to minimize any potential impairment, the collection tubes should be kept in a tube warmer before being connected to the collection system.

14.3 Indication

Typically, a mature preovulatory COC is characterized by the presence of radiating corona cells surrounded by the expanded mass of CCs. The quality and the degree of expansion of these cells do not always represent a reliable index of oocyte maturity and are mostly dependent on the type of ovarian stimulation protocol used [12, 13, 19–21].

About 34–38 h after triggering ovulation, oocytes are supposed to be mature at the metaphase II stage, although it is possible to run into an immature oocyte at the germinal vesicle or metaphase I stage even in an expanded and mucified cumulus. Therefore, during ART procedures, cumulus cells surrounding the oocyte are removed to improve oocyte evaluation and manipulation.

Since the introduction of assisted fertilization techniques such as PZD (partial zona dissection), SUZI (subzonal insemination), and finally ICSI (intracytoplasmic sperm injection) [22], the removal of the surrounding cumulus and corona cells became a crucial step before fertilization. It allowed an unequivocal evaluation of oocyte nuclear maturity and identification of the first polar body in the perivitelline space that indicates a successful completion of meiosis I with arrest at the MII stage of development. Moreover, direct observation of oocyte morphology, including the extracytoplasmic components, is feasible only after denudation of its cumulus and corona layers.

In order to avoid sperm DNA contamination, ICSI has been indicated as the preferred insemination method when preimplantation genetic diagnosis is required. To this end, complete and meticulous removal of somatic cells is similarly of crucial importance, since extraneous DNA from maternal cumulus cells may also represent a potential source of contamination if a PCR-based technology is used [23].

14.4 Equipment and Procedure

The cumulus and corona cells are removed prior to any micromanipulation procedure by exposure of the COCs to a combined enzymatic and mechanical treatment that causes the breakdown of the hyaluronan-based matrix surrounding COCs and disperses the cumulus cells from oocytes [24]. Since hyaluronic acid is a major component of the mucified cumulus mass that surrounds the mature oocyte, hyaluronidase is employed for enzymatic removal of these cells. The type and concentration of this enzyme will be discussed later.

The procedure is usually performed under a stereomicroscope in different wells or microdrops containing buffered medium, usually covered by oil, on a warmed thermo plate.

A limited number of COCs are normally placed into the first well/droplet containing the enzyme to disperse the cumulus cells (Fig. 14.1a). To enhance enzymatic removal of the cumulus and corona cells, the stripping of cumulus cells is performed by pipetting repeatedly the oocytes through a glass/plastic pipette with an inner diameter of approximately 250 μm for up to 30–40 s.

Once initial cell dissociation is observed (Fig. 14.1b), oocytes are moved to the second well/droplet containing buffered medium, taking care to carry over a minimum amount of enzyme. Further denudation is then carried out to remove the corona cells by glass or plastic denuding pipettes with decreasing inner diameters (170–130 μm) (Fig. 14.1c). An accurate washing of denuded oocytes is then required to finally wash out the enzyme.

It has been proposed that a two-step denuding procedure involving an incubation period after oocyte exposure to the enzyme and before the mechanical step [25] may expedite the removal of cumulus cells while minimizing mechanical-induced oocyte damage. After partial removal of the cumulus cells by a short exposition to buffered medium containing the enzyme, the oocytes are rinsed in pre-equilibrated culture medium and placed back into the incubator for 30 min. This further step leads to a weakening of the hyaluronan matrix allowing an easier cumulus cell removal by gently pipetting oocytes with a 150-μm-inner-diameter stripper.

After denudation, the oocytes can be examined under an inverted microscope to assess integrity and the stage of maturation.

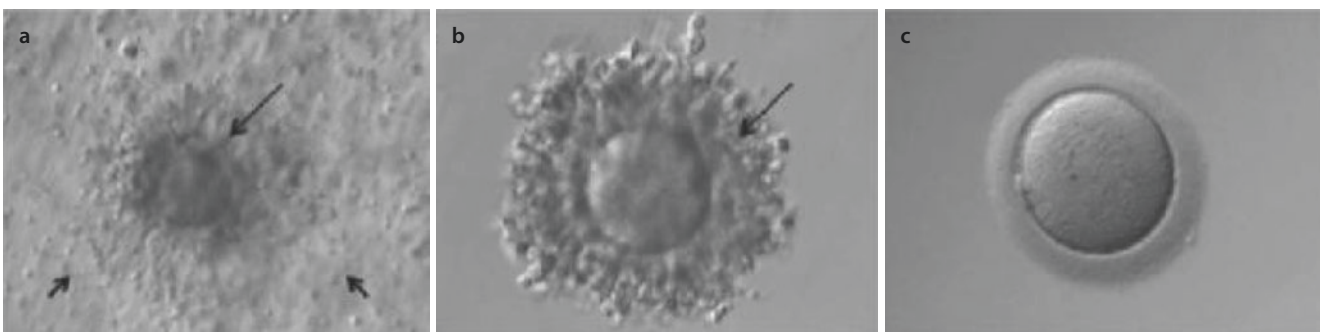

Fig. 14.1 Oocyte denudation steps. **a** Oocyte surrounded by the corona radiata (*short arrow*) and cumulus cells (*long arrow*); **b** oocyte after the enzyme-induced dispersion of cumulus cells; **c** MII denuded oocyte

14.5 Timing

The oocyte denudation is usually performed between 37 and 40 h post-hCG administration and immediately before the insemination. Already in 1982, Trounson et al. [26] postulated that preincubation of cumulus–corona–oocyte complexes before conventional insemination was beneficial to the accomplishment of complete oocyte maturation and assured high rates of fertilization and embryo development in vitro.

However, no differences were reported between the effect of early (1–2 h after retrieval) and late (5–6 h after retrieval) denudation and injection on early embryo development [27]. The authors assumed that the stimulation protocol involved in the study allowed a well-synchronized nuclear–cytoplasmic maturation hiding the potential contribution of the cumulus–corona complex preincubation.

To evaluate whether the timing of ICSI has an effect on ICSI outcome, we analyzed the possible effects of preincubation of CCOC on fertilization and embryo development rates after ICSI [28]. In agreement with Ho and coauthors [29], we observed that a preincubation period of at least 3 h between oocyte retrieval and ICSI could improve the fertilization rate and embryo quality. This time might be necessary for some oocytes to reach full cytoplasmic maturity, leading to a higher activation rate upon microinjection.

Our observations have been challenged by Yanagida et al. [30], who showed that oocytes retained sufficient potential for fertilization between 1 and 9 h after oocyte collection. On the other hand, they observed that an extended preincubation time might be responsible for impaired embryo viability.

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14.6 Oocyte Evaluation

In stimulated cycles, sibling oocytes belonging to the same cohort may differ greatly in their developmental potential. While morphological assessment of preimplantation-stage embryos is a basic element of the laboratory routine, the evaluation of the retrieved oocytes is rather rough and superficial. However, oocyte quality is a key limiting factor, holding a crucial role in fertilization, but affecting also the following embryo development [31]. Therefore, a proper selection of the most developmentally competent oocytes to inseminate would be valuable to improve the efficiency of ART treatment. However, due to the complexity of the oocyte maturation process, a comprehensive assessment of oocyte quality would require a detailed and noninvasive analysis of different key markers.

14.6.1 COC Morphological Assessment

Several scoring systems for cumulus–corona–oocyte complex evaluation have been introduced aiming to predict the nuclear maturity of the overshadowed oocytes and identify the proper timing for insemination [20, 32–37].

Early studies from Rattanachaiyanont et al. [20], performed on oocytes scheduled for denudation and insemination by ICSI, reported no correlation between oocyte–corona–cumulus complex morphology and nuclear maturity, fertilization rate, and embryo cleavage. On the other hand, other authors reported that COC scoring is related to fertilization and pregnancy rates [36] as well as to blastocyst quality and development [37].

According to the Alpha-ESHRE consensus document, there is not enough validated evidence to support the existence of a correlation between the appearance of the COC and embryo developmental competence; however, a binary score (0 or 1) with a "good" COC (score 1) defined as having an expanded cumulus and a radiating corona can be used as reference (38).

It has also been suggested that the presence of COC anomalies such as blood clots or amorphous clumps may be an index of a suboptimal follicular maturation and may impair embryo ability to develop to blastocyst stage [39, 40]. When these oocytes are denuded of their cumulus cells, a significant alteration in cytoplasmic texture has been highlighted that may be related to the reduced fertilization rate obtained when those oocytes are used for insemination [40]. Variations in temperature and pH as well, as reactive oxygen species induced by the presence of blood clots, are pointed to be at the origin of the compromised competence of those oocytes [34].

14.6.2 Oocyte Cytoplasmic and Extracytoplasmic Evaluation

It is generally recognized that a typical human metaphase II oocyte should have a round, clear zona pellucida; a small perivitelline space containing a single not fragmented polar body; and a pale, moderately granular cytoplasm not containing inclusions [41–48].

However, the majority of the oocytes retrieved after ovarian hyperstimulation exhibit one or more deviations from the described "ideal" morphological criteria [41, 44–50]. This is true also for oocytes obtained from proven fertile donors [51].

14.6.3 Nuclear Maturity

During oocyte development within the follicle, the nucleus is arrested at the prophase of the first meiotic division. Oocyte nuclear maturation consists in the resumption of the first meiosis, going through the germinal vesicle (GV) breakdown, first meiotic spindle (MS) formation, chromosomal segregation, and IPB extrusion. At this stage, the maturation process is not ended yet, and the oocyte has still to enter the second meiotic division where a second arrest occurs at metaphase II (MII) stage. This stage is characterized by the presence of the MS of the second meiotic division located at the oocyte periphery with one pole attached to the cell

cortex. This highly dynamic structure is formed by microtubule, with the chromosomes condensed and aligned at the equatorial region [52–56]. After insemination, this structure plays a crucial role in chromosomal disjunction and segregation and in the correct completion of meiosis and fertilization. MS alteration and dysfunction lead to unbalanced disjunction and/or non-disjunction of chromatids, chromosome scattering, and the formation of aneuploid embryos [54, 57, 58].

The presence of IPB in the perivitelline space is a marker of oocyte meiosis resumption, but not an evidence of MII stage achievement. It is only observing the presence of the MS of the second meiotic division that the oocyte nuclear maturation can be definitely assessed. Additional information on oocyte nuclear status can be acquired through the use of polarized light microscopy combined with software for image processing for the noninvasive visualization of the MS and other oocyte birefringent structures [59–62]. These systems consist of a microscope equipped with a green filter and a polarizer generating circularly polarized light. Highly ordinate and well-organized structures, such as the parallel-aligned microtubules composed of the oocyte MS, are able to shift the vibration plane and retard the polarized light. The induced retardance can be measured (nm), and this parameter is directly related to the microtubule density [60, 63, 64].

These systems allow to assess the position and appearance of the spindle and to recognize those oocytes that have not completed the first meiotic division and are still in telophase I [65–67]. At this time of the cell cycle, the oocyte cytoplasm and the cytoplasm of the forming IPB are not yet completely separated by cytokinesis. The MS appears to be interposed between the two separating cells, with its long axis corresponding to the axis connecting the center of the two cells. An oocyte at this stage is usually classified as a "mature MII" when observed with the conventional light microscopy. Although at this stage the presence of the IPB is evident in the perivitelline space, chromosome segregation is not completed. This condition normally takes 75–90 min. The MS then disappears in late telophase I, reappearing only 40–60 min later [68]. Therefore, at least in some cases, the absence of MS signal is due to the oocyte maturation process. However, oocyte exposure to suboptimal culture conditions during micromanipulation can contribute to MS disassembly [69]. Finally, the percentage of oocytes with detectable MS is also related to the time elapsed from hCG administration and is higher after 38 h [70]. In general, it is expected that at least 80% of oocytes recovered following ovarian stimulation are MS positive when viewed by polarized light microscopy.

When studying the MS, some factors must be taken carefully into account in order to keep under control those that may interfere with the visualization of the spindle (▶ Box 14.1). First is temperature control during oocyte handling [71, 72]. Second, the absence of MS may be due to unfavorable culture conditions and environmental changes [73]. Third is the MS orientation [74–76]: oocyte rotation by using a micropipette allows the correct orientation of the MS making it visible at the polarized light, with the long axis oriented perpendicular to the viewer's 66 axis [64, 74, 77].

Box 14.1 Parameters that should be considered during MS visualization with polarized light microscopy

1. Presence/absence of MS, which may be due to:
   1. Laboratory factors
      1. Temperature instability
      2. Chemical and physical stress during handling and culturing
      3. Correct oocyte rotation during visualization
   2. Oocyte maturation, which is related to:
      1. Time elapsed from hCG administration
      2. Interval between the first and second meiotic divisions
   3. Maternal factors (i.e., age)
2. Position and appearance of MS:
   1. Allow identification of immature oocytes (late telophase I)
   2. Allow assessment of IPB/MS relative position, which affects oocyte fertilization rate and the orientation of the first cleavage plane
   3. Allow the correct orientation of MS during ICSI

14.6.4 Cytoplasmic Aspect

To acquire full competence for subsequent fertilization and development, the oocyte has to undergo nuclear and cytoplasmic maturation in a coordinated manner. Disturbances or asynchrony of these two maturation processes is believed to result in a variety of oocyte morphological abnormalities [78–80]. The presence of cytoplasmic (vacuoles, refractile bodies, increased cytoplasmic granularity, smooth endoplasmic reticulum clusters) and extracytoplasmic (abnormal zona pellucida, large perivitelline space, or abnormal, fragmented, or degenerated IPB) defects may potentially reflect oocyte developmental impairments [41, 42, 48, 50, 81, 82].

However, a systematic analysis of 50 papers aimed to evaluate the predictive value of morphological assessment of MII phase oocytes has produced contradicting results [83]. None of the investigated features were found to have prognostic value in terms of oocyte viability and developmental competence.

This outcome may be due to the heterogeneity of the grading systems employed for oocyte morphological assessment and to the different outcome parameters assessed.

According to the Istanbul consensus workshop on embryo assessment [38], oocytes displaying cytoplasmic smooth endoplasmic reticulum (SER) aggregates should be managed with caution, because of an increased risk of an abnormal outcome. This conclusion was based on previously reported cases of Beckwith–Wiedemann syndrome [84], diaphragmatic hernia [85], multiple malformations [86], and ventricular septal defect [87] after transfer of embryos originated from SER-positive oocytes.

However, few years later, deliveries of normal healthy babies after the transfer of embryos derived from MII oocytes with visible SER [88] have been reported. Afterward, a systematic review analyzing the clinical outcomes for SER-positive oocytes could not demonstrate the existence of a clear association between SERs and fetal malformations. The birth of healthy babies from SER-positive embryos is encouraging and might lead to a revision of the current consensus in the future. In the meanwhile, oocytes displaying these abnormalities should be approached with caution, and the deriving embryos should be transferred only when no alternative viable embryos are available [89].

14.6.5 Extracytoplasmic Structure Evaluation

Extracytoplasmic peculiarities are classified as fragmented I polar body, abnormal I polar body (large and/or degenerated), abnormal zona pellucida (with sets and/or thick and/or dark), large perivitelline space, and abnormal oocyte shape (oval).

The Alpha-ESHRE consensus document stated that there is insufficient evidence to support any specific prognosis associated with these morphological abnormalities, with the exception of large polar body and giant oocytes [38].

The consensus suggested that oocytes with a remarkably large first polar body should not be considered for insemination, due to an increased risk of oocyte aneuploidy. In our setting, the presence of an abnormal first polar body in the metaphase II oocytes analyzed was relatively rare (4.4%). According to animal experiments [90], it has been postulated that the emission of an abnormally large polar body is due to the inability of the meiotic spindle to migrate correctly at the very periphery of the cell. It is, in fact, the position of the main axis of the meiotic spindle that dictates the orientation of the cleavage furrow. A centrally located meiotic spindle would lead to the formation of enlarged polar body [91]. The polar body morphology, and in particular large polar bodies, may be thus considered a marker of oocyte maturation disturbances [90, 91].

Special attention should also be focused on giant oocytes. Although the occurrence of these oocytes is relatively rare after ovarian hyperstimulation, the use of these cells for in vitro fertilization is potentially dangerous. It has been described that all embryos generated from giant oocytes are chromosomally abnormal, but they may have a normal cleavage and development to blastocyst stage [92]. The transfer of these embryos could thus increase the risk of undesired miscarriages [92].

14.6.6 Noninvasive Analysis of CCs

Efforts to date are directed to the identification of new reliable biomarkers of oocyte quality in order to compensate for the inadequacy of the COC morphological assessment [93, 94].

Follicular fluid, granulosa, and cumulus oophorus cells may offer novel biomarkers due to their close relationship to the oocyte. The bidirectional communication between the oocyte and CCs, essential for the acquirement of oocyte competence [31], can be investigated through noninvasive analysis of CCs (pattern of gene expression or protein synthesis). Given the complexity of the follicular differentiation process, a comprehensive evaluation of oocyte quality may involve the use of a combination of multiple markers.

Several authors have focused their attention to CC gene expression analysis as a noninvasive tool in predicting oocyte competence [95–103]. The main approach used to study CC gene expression is microarray analysis, to simultaneously evaluate the different expression of a large number of genes, followed by quantitative real-time polymerase chain reaction confirmation of CC transcriptome.

Although these results offer a set of potential biomarkers that could be useful for oocyte selection, the final end points of the studies in literature are quite different, generating some discrepancies in the results. To date, more evidence is needed in order to define a CC gene expression pattern that can be considered as noninvasive marker of oocyte quality.

14.7 Possible Damage to the Oocyte

14.7.1 Oocyte Parthenogenetic Activation

In mouse and human oocytes, chemical agents such as ethanol and Ca^{2+} ionophore as well as electrical field stimulation may initiate a series of events leading to the release of the meiotic arrest at metaphase II and consequent cell divisions of the activated oocyte. Although human oocytes seem to be less susceptible to artificial activation in comparison to other mammalian oocytes, several factors related to IVF procedures, such as the high vacuum pressures used for follicle aspiration, the high concentration of hyaluronidase used for cumulus cell stripping, as well as oocyte pipetting, may activate mature oocytes and induce parthenogenetic activation [104, 105].

When ICSI has been introduced, a concentration of 760 IU/ml of hyaluronidase was used to denude oocytes, but high parthenogenetic activation rates (17%) were observed [105]. Van Steirteghem et al. [106] were able to reduce the activation rate to 3% by employing lower concentration (78 IU/ml) of enzyme. Moreover, Joris et al. [107] investigated whether a further reduction of hyaluronidase concentration might influence oocyte intactness and its ability to fertilize and support embryo development. Although no significant effect on ICSI outcome was observed, a significant lower degeneration rate was registered when a concentration of 10 IU of enzyme per ml was used. This finding would justify the use of lower concentration of enzyme.

14.7.2 Meiotic Spindle

A less perceptible effect but likewise harmful to the oocyte is represented by the potential displacement of the polar body

following the denuding procedure. Notwithstanding the intrinsic ability of the polar body to move along the plasma membrane of the oocyte, the mechanical stripping of cumulus cells may impair the frame of microtubules and the actin filament organization, causing the break of the cellular bridges that connect the polar body to the oocyte. This would result in an IPB displacement from its original extrusion site [74].

The use of polarized light microscopy may be helpful in the assessment of the position of the MS in regard to the IPB. MS position at the very periphery of the cell, attached to the oolemma cortex [108], dictates the orientation of the cleavage furrow and thus the site of IPB extrusion. According to different studies, moderate degrees of IPB/MS deviation do not seem to affect ICSI outcome, in terms of fertilization and embryo development rates [74, 109]. However, in our laboratory, we found that a mechanical stress able to induce a dislocation of the IPB ≥90° with respect of the MS position is related to a lower fertilization rate [74]. MS dislocation has also been reported to affect embryo development since its position determines the correct orientation of the first cleavage plane and therefore of the animal vegetal pole [77]. It is thus advisable to perform oocyte denudation in a gentle manner.

Besides the hypothetical effect on ICSI outcome, a possible drawback of IPB displacement is the potential injury to the MS during microinsemination. In fact, when performing ICSI, the oocyte is maintained in a fixed position where the IPB is placed at 90° from the injection pipette entry site. Displaced IPB may thus expose the MS to the pipette passage during sperm injection and hence to mechanical damage. In view of this fact, the use of polarized microscopy was considered helpful during ICSI to allow the correct orientation of the MS (and not of the IPB). However, the usefulness of this technology for routine ICSI, together with the additional time and expense, is still questionable, since some studies have shown comparable fertilization and development rate of human oocytes subjected to ICSI regardless of the position of the spindle relative to PB1 [109, 110].

Moreover, it has been widely shown that oocyte exposure to even slight temperature variations dramatically injures the MS structure by inducing microtubule disassembly [73, 111–114]. In fact, MS microtubules are highly sensitive to chemical and physical stress that may occur during oocyte retrieval and handling. Thermal stability is therefore necessary during oocyte denudation, observation, and manipulation.

14.7.3 Mechanical Stress

In order to avoid damage to the oocytes, meticulous care should be taken during the mechanical removal of cumulus cells. In fact, excessive or vigorous pipetting of the oocyte may result in zona pellucida fracture and even oocyte degeneration. The stripping of the cumulus cells should be performed gradually by employing decreasing inner diameter denuding pipettes. The use of a stripper with a diameter smaller than 140 μm should be avoided since it may induce oocyte deformation and damage the oocyte cytoskeleton impairing its ability to sustain correct pronuclear formation.

14.8 Enzyme Type and Concentration

14.8.1 Hyaluronidase or Coronase

Traditionally, the denuding procedure involves a bovine-derived hyaluronidase. Bovine testicular hyaluronidase is an endo-β-*N*-acetyl-D-hexosaminidase that catalyzes the hydrolysis of hexosaminidic linkage contained in hyaluronic acid. Although purified preparations of the enzyme are commercially available, the possibility of animal-derived pathogens or contaminant transmission cannot be excluded [115]. The use of a plant enzyme preparation containing citric acid and pagane as well as NaCl and phosphate (coronase) has been proposed as an alternative to animal-extracted hyaluronidase. Because of the different action mechanisms of coronase that acts by chelating Ca^{++} ions and inducing a degradation of glycoprotein granules of the hyaluronic matrix, a longer exposure time to the enzyme is required [116]. However, coronase exhibited similar performance in terms of oocyte integrity and competence preservation when compared to conventional hyaluronidase. Moreover, because of the absence of any human or animal compounds, it may be more suitable for the clinical practice [116].

14.8.2 Enzyme Concentration

In order to minimize any potential toxic effect on oocytes, a reduced enzyme concentration as well as shorter exposure time is advisable. As already mentioned, lower concentrations of the enzyme such as 80 IU/ml, which is generally used in the clinical practice, significantly decrease the rate of parthenogenesis. A concentration as low as 10 IU/ml in combination with a denuding pipette of 1000 μm diameter has also been shown to be able to denude mature oocytes efficiently [25].

14.8.3 Animal-Derived or Recombinant Human Hyaluronidase

Several concerns arose from the clinical application of the animal origin enzyme that may be affected by a reduced purity and standardization. Human recombinant hyaluronidase (Cumulase) has been proposed to bypass problems and concerns associated with the animal-derived enzyme. De Vos et al. [117] have conducted a randomized sibling-oocyte study in order to validate the effectiveness of recombinant human hyaluronidase versus bovine-derived hyaluronidase, observing comparable results in terms of oocyte survival and fertilization. A following study [118] confirmed these findings with a significantly increased fertilization rate and decreased oocyte damage rate when the recombinant form of the enzyme was involved.

Therefore, the human recombinant form of the enzyme may represent a safer, quality-controlled, but likewise effective alternative.

14.9 Influence of Microenvironmental Conditions

A general principle for the physical environment is to keep it free of any insults. The handling of oocytes during the entire IVF procedure should be carried out under conditions of constant pH and stable temperature.

In order to prevent the evaporation of the medium and minimize the fluctuations of pH and temperature, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES)-buffered media and temperature-controlled humidified chambers are employed to maintain these parameters in a physiological range.

14.9.1 Temperature

We have already affirmed that temperature fluctuations likely associated with the handling of oocytes may impair the microtubular system. Changes in spindle organization have been observed in human mature oocytes exposed to room temperature even for only few minutes [112]. Notwithstanding the ability of the meiotic spindle to reassemble when the temperature is reestablished, the risk of aneuploidy occurrence is increased after a temperature-induced depolymerization [112].

Because of this extreme meiotic spindle sensitivity to temperature changes, all the equipment in use (Petri dishes and Pasteur pipette) should be prewarmed at 37^0 C. In order to maintain a constant temperature in the droplets, the working areas (hood and microscope) and the thermo plate must be calibrated regularly. However, it is very difficult to maintain constant temperature without the use of specific chambers.

14.9.2 CO_2 Concentration

Dale et al. demonstrated that the baseline intracellular pH (pHi) of human egg is 7.4 ± 0.1 [119]. Unlike germinal vesicle that is endowed with a robust mechanism of pH regulation, denuded metaphase II oocytes are deficient of HCO_3^-/Cl^- and Na^+/H^+ functioning exchangers and do not have the ability to control and regulate intracellular pH [120]. It is only 2–3 h after fertilization that the oocyte begins to recover the exchanger activity and the consequent ability to regulate its pH. HEPES-buffered media are usually employed during oocyte handling outside the incubator in order to preserve oocyte homeostasis and avoid pHi fluctuations that may impair developmental competence. However, media containing bicarbonate/CO_2 buffers would be preferable, since requiring controlled chambers to maintain a 5–7% CO_2 atmosphere avoids the use of HEPES and thus the increased risk of toxicity to the oocyte. In fact, the low percentage of CO_2 entails a low percentage of HCO_3^- in the media, essential to the functionality of HCO_3^-/Cl^- exchanger and thus the HCO_3^--dependent mechanism that oocytes utilize to defend against acidosis. The use of combined multiple buffers, such as HEPES, MOPS, and DIPSO, into a single medium has been proposed to enhance the buffering capacity over a range of temperatures while simultaneously reducing the absolute concentration of individual buffers and then related toxicity concerns [121].

14.9.3 O_2 Concentration

Several IVF procedures entail an increase in oxidative stress that may be detrimental to the embryo. Although several physiological events, such as ovulation, fertilization, as well as the implantation process, are modulated by a reactive oxygen species (ROS) formation [122–124], a considerable increase in ROS concentration exceeding the oocyte antioxidant defenses may result in cellular structural and functional alterations. It has already been shown that during their journey along the female reproductive tract, embryos encountered a decreasing oxygen gradient. Moreover, oxygen levels in the oviduct and uterus of several species are considerably lower than in the atmosphere (1.5–6% vs. 21%). However, historically, most IVF laboratories have cultured embryos under atmospheric oxygen concentrations, probably because of the additional expenses required to perform culture at a lower oxygen concentration.

There are enough validated evidence for recommending the use of low oxygen tension (5%) within the incubation atmosphere to improve embryo development and potential to implant [126–148]. However, this benefit was observed only for embryos cultured and transferred at the blastocyst stage, and not for those at the cleavage stage. Even if the precise O_2 level may be developmental stage-dependent, the low oxygen level should be a principle for all mammalian embryo culture systems including humans.

14.10 Incomplete Denudation

In support of a positive effect of cumulus–corona cells on oocyte development, it was proposed that incomplete denudation of oocytes prior to ICSI improves embryo quality and development since it may assist embryonic metabolism, either stimulating gene expression [15] or reducing oxidative stress [16]. A study involving sibling oocytes investigated the actual effect of homologous adhering cumulus cells, resulting in improvement in terms of preimplantation development when oocytes were cultured with attached cumulus cells [149].

Similar findings were obtained in previous studies suggesting that the presence of cumulus cells allows better nuclear as well cytoplasmic in vitro maturation, particularly in oocytes that were immature at the time of denudation [150, 151].

14.11 Future Direction and Improvement

The involvement of automated systems in IVF procedures is attracting increasing attention. A micro-fluidic device has been proposed to remove cumulus cells automatically, aiming to minimize physical stresses related to this procedure [152]. The system entails pressure-driven flow to obtain a correct positioning and transit of COC into a micro channel that allows yielding predictable cumulus removal. Although further studies are required to assess the impact of these procedures on embryo health, several efforts are focusing on the development of a single integrated automated system skilled to perform all the steps from oocyte retrieval to insemination and embryo culture.

Review Questions

1. What are the indications to perform oocyte denuding?
2. What is the best timing to perform oocyte denudation?
3. Which morphological features should an "ideal" human metaphase II oocyte show?
4. What are the typical enzymes used to perform oocyte denudation in clinical practice?
5. Which microenvironment conditions should be properly and finely controlled during oocyte handling?
6. What are the most promising forthcoming devices that could be introduced in the near future to perform oocyte denudation?

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Embryo Culture Methods

Contents

Interrupted or Uninterrupted Culture to the Blastocyst Stage in a Single Medium

Don Rieger and Klaus E. Wiemer

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Learning Objectives

- To understand the history of the development of culture media for human embryos
- To understand the characteristics and advantages of interrupted or uninterrupted culture in a single medium
- To understand the importance of laboratory air quality and other aspects of the culture conditions to the use of uninterrupted culture
- To know that each laboratory must make a careful comparison between interrupted and uninterrupted culture before adopting uninterrupted culture as routine practice

15.1 Introduction

The objective of embryo culture, as part of human-assisted reproduction, is to provide a suitable environment for the embryo to grow and develop from fertilization through to the appropriate stage for transfer into the uterus or cryopreservation. Although embryo transfer was originally done at the cleavage stage (Day 2 to Day 3), it has become clear that it is preferable to transfer at the blastocyst stage (Day 5 or 6), for a number of reasons. First, in spontaneous conception, the human embryo does not normally pass into the uterus until the morula to early blastocyst stage, and the uterus is likely not well equipped to support the cleavage-stage embryo. Second, gonadotrophin stimulation of the ovary to provide an increased number of oocytes results in very high circulating concentrations of estrogen that lead to abnormal development and functional activity of the uterus. Delaying transfer until the blastocyst stage allows the uterus to return, at least partially, to a more normal condition. Third, culture to the blastocyst stage allows for more extensive evaluations of the embryos, for the purposes of selection (and to a certain extent, self-selection) of the best one(s) for transfer. Before the major onset of expression of the embryonic genome, on Day 3, the development of the embryo is largely driven by the proteins and messenger RNA present in the oocyte. Evaluation of the cleavage-stage embryo provides little or no opportunity to evaluate the morphological, metabolic, or functional activity arising from the embryonic genome. Most notably, the de-selection of aneuploid embryos by preimplantation genetic screening (PGS) is much more effective at the blastocyst stage (trophectoderm biopsy) than at the cleavage stage (single blastomere biopsy).

Without question, the implantation rate for blastocyst transfer is superior to that for cleavage-stage transfer, so much so that high pregnancy rates can be achieved with the transfer of single blastocyst, with a very low incidence of multiple births. When combined with vitrification and single transfer in a subsequent unstimulated cycle, and 24-chromosome trophectoderm biopsy PGS, clinical pregnancy rates of greater than 60% are achievable, with almost no multiple births [1–3]. This must be considered as the future of human-assisted reproduction treatment (ART).

15.2 A Brief History of the Development of Embryo Culture Media

Defined tissue culture media began with Ringer's [4] simple salt solution containing NaCl, KCl, $CaCl_2$, and a low concentration of sodium bicarbonate ($NaHCO_3$), based on the constituents of blood serum, for the study of the beating frog heart, in vitro. Various modifications were made for the study of specific tissues (e.g., [5, 6]). Most notably, Krebs and Henseleit [7] increased the concentration of sodium bicarbonate to 25 mM (Krebs-Ringers bicarbonate, KRB). This 25 mM bicarbonate concentration is conventionally used with 5% CO_2 in many tissue culture media to produce a physiological pH.

Defined culture of mammalian embryos began with Whitten [8], who used KRB supplemented with 5.55 mM glucose and showed that eight-cell mouse embryos would develop to the blastocyst stage. In a landmark study, McLaren and Biggers [9] cultured eight-cell mouse embryos to the blastocyst stage in Whitten's medium and produced live young when they were transferred to recipient mothers. Whitten [10] added lactate and could culture outbred two-cell mouse embryos, but not zygotes, to the blastocyst stage, which led to the concept of the "two-cell block" to mouse embryo culture. Subsequent modifications of the concentrations of energy substrates by Brinster [11] and Whittingham [12] resulted in BMOC2 and M16, respectively. These media were widely used for mouse embryo culture, but, except for inbred strains of mice, they could not overcome the two-cell block. Blocks to development in vitro were similarly found for hamster (2 cells), cattle (8–16 cells), pig (4–8 cells), and human (4–8 cells) embryos, all approximately coincident with the major onset of expression of the embryonic genome (see [13]). Chatot et al. [14] were able to culture mouse embryos through the two-cell block with CZB medium, a modified version of BMOC2 with no glucose, 1.0 mM glutamine, and 0.1 mM EDTA. Although a significant step forward, it led to the mistaken idea that glucose was inhibitory to early embryo development (see [15]).

Embryo culture media based on simple salt solutions were followed by the "back to nature" approach to the formulation of media using the measured concentrations of the components of oviduct and uterine fluids: SOF, based on sheep oviduct fluid [16]; B2, based on cattle oviduct and uterine fluids [17]; HTF, based on human oviduct fluid [18]; MTF, based on mouse oviduct fluid [19]; and G1/G2, based on human oviduct and uterine fluids [20].

As a result of the measured differences in the composition of oviduct and uterine fluids and measured changes in the metabolic activity of embryos during early development, Gardner and Lane [21] suggested that "in order to optimize mammalian embryo development in culture, sequential media are required, each designed to meet the changing requirements of the developing embryo." In this scheme,

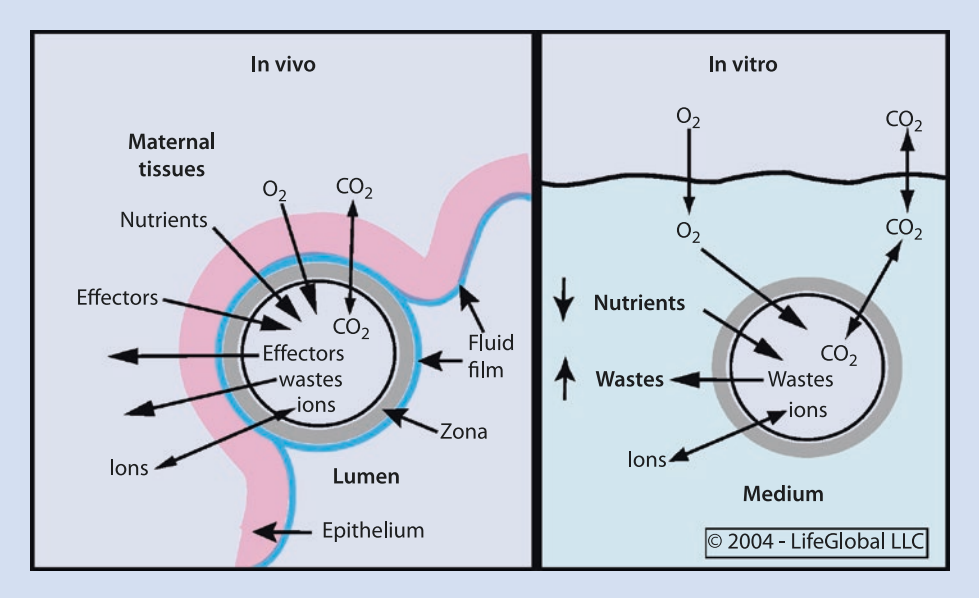

Fig. 15.1 A comparison of the interaction of an embryo with its environment, in vivo and in vitro. (Reproduced with the permission of Life Global LLC)

embryos are cultured from the zygote to the eight-cell stage (Day 1 to Day 3) in a medium based on the oviduct fluid and then in a second medium, based on the uterine fluid, for culture onward to the blastocyst stage (Day 3 to Day 5 or 6). Although this seems logical, this "back to nature" approach relies on several questionable assumptions.

First, the measurements of the components of the oviduct and uterine fluids are highly variable [22] and almost certainly subject to physiological inductance. Second, such measurements are of the overall composition of the tract fluids and not the microenvironment around the embryo. Third, as shown in Fig. 15.1, the physical and chemical environment of the embryo in vivo is completely different from its environment in vitro. In vivo, the embryo is surrounded by a thin layer of fluid and is in close contact with the maternal tissues, allowing rapid exchange of nutrients, gases, wastes, and effectors between the embryo and the mother. In contrast, in vitro, the embryo is bathed in a relatively large pool of fluid in which the nutrients are continually decreasing and the waste products are continually increasing during culture. Clearly, the stresses on the embryo in vitro are very different from those in vivo, and culture media must be designed to optimize embryo development under in vitro conditions.

Using a very different approach to the development of embryo culture media, Lawitts and Biggers [23, 24] applied the principles of simplex optimization to determine the optimal concentration of each component. They began with a medium based on M16 and CZB, containing NaCl, KCl, KH_2PO_4, $MgSO_4$, lactate, pyruvate, glucose, bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), and glutamine. In a series of test cycles, they varied the concentration of each component and evaluated each combination for the ability to support the development of mouse zygotes through the two-cell block. The component concentrations in each cycle were based on the effectiveness of the medium in the previous cycle. In essence, the embryo defined the formulation of the successive test media, and this is often referred to as the "Let the Embryo Choose" approach to medium development.

A total of 20 such cycles resulted in the formulation of simplex optimization medium (SOM), which was marked by a low NaCl concentration and was able to overcome the mouse two-cell block [24]. Subsequent modifications included increasing the concentration of KCl to produce KSOM medium [25], and addition of all 20 common amino acids to produce KSOMaa [26–28]. Biggers et al. [29] and Summers et al. [30] subsequently showed that the development of mouse embryos were better when the medium included the glycyl-glutamine dipeptide, compared to either glutamine or alanyl-glutamine.

In addition to many studies on mouse embryos, KSOM, with or without amino acids, has been shown to support the development of cattle [31], rabbit [32], rhesus monkey [33], pig [34], and rat [35] embryos from the zygote to the blastocyst stage. For more detailed and extensive reviews of the history of the development of embryo culture media, see Biggers [36], Hammer [37], Summers and Biggers [22], and Cohen and Rieger [38].

15.3 Culture of Human Embryos in a Single Medium from the Zygote to the Blastocyst Stage

Based on the success of KSOMaa, as a single medium, for culture of mouse embryos and embryos of other species, Biggers and Racowsky [39] compared the development of human embryos in KSOMaa (Days 1–5) with the development in the sequential pair of P1 (Days 1–3) and CCM (Days 3–5). The

Table 15.1 Characteristics of approaches to embryo culture from zygote to blastocyst

Characteristic	Sequential media interrupted	Single medium interrupted	Single medium uninterrupted
Leaves embryos undisturbed	No	No	Yes
Accumulated endogenous growth factors	Lost	Lost	Left in place
Replacement of essential nutrients	Yes	Yes	No
Accumulated toxins	Removed	Removed	Left in place
Relative stress to embryos	High	Moderate	Low
Required quality control	Two media	One medium	One medium
Relative labor intensity	High	Moderate	Low
Relative cost	High	Low	Low

Adapted from Biggers and Summers [47]

development to the expanded blastocyst stage was not different between KSOMaa and the P1/CCM sequential media pair. Transfer of nine blastocysts from KSOMaa culture resulted in the birth of five babies.

Macklon et al. [40] compared the development of human embryos in their Rotterdam medium (17:3 mixture of Earle's salts and Ham's F-10) either uninterrupted or with a change to fresh medium on Day 3, against the G1/G2 sequential media pair. The development to blastocyst and implantation rate were not different among the three treatment groups.

Many subsequent studies have shown a single medium (based on KSOMaa) to be as effective as, or more effective than, various sequential media pairs, for the development of human embryos to the blastocyst stage and for implantation. Examples include Nanassy et al. [41], Sepulveda et al. [42], Carrillo and Yalcinkaya [43], Wirleitner et al. [44], Summers et al. [45], and Sfontouris et al. [46]. In these studies, the single medium was used in two steps, with a change to fresh medium on Day 3, in parallel with the change from the cleavage medium to the blastocyst medium in the sequential media.

Biggers and Summers [47] have suggested that a single-step (uninterrupted) culture may be preferable to both sequential culture and two-step (interrupted) culture in a single medium, for culture of human embryos from the zygote to the blastocyst stage (Table 15.1). Practical advantages of uninterrupted culture include reduced quality control procedures, labor, and cost. Physiological advantages of uninterrupted culture include the possibility of accumulation of endogenous growth factors and a decrease in stress to the embryo. Direct comparisons have shown that uninterrupted culture in a single medium can be as effective as interrupted culture in the same single medium, or culture in sequential media [48–53]. Uninterrupted culture is of particular interest for time-lapse evaluation of embryo development, and this too has been shown to be effective with a single medium [54–57].

It must be noted that uninterrupted culture increases the risk of accumulation of embryo-toxic volatile organic compounds (VOCs) from the culture dishes, laboratory gases, and the environmental air into the culture oil and then the culture medium. Studies have shown that laboratory air quality is a critical aspect of embryo culture and that common VOCs such as benzene and aldehydes can accumulate in the oil and medium used for embryo culture [58–62]. Uninterrupted culture increases the time for potential accumulation of VOC and hence the possibility of deleterious effects on the embryos. Consequently, the laboratory air quality should be critically evaluated, and a careful comparison of the effectiveness of uninterrupted vs. interrupted culture should be made before introducing uninterrupted culture as general practice in any ART laboratory.

15.4 Procedures for Interrupted or Uninterrupted Embryo Culture in a Single Medium

The following describes the materials and methods used for uninterrupted culture in a single medium at POMA Fertility.

15.4.1 Culture Media

For embryo culture, we use washed oil, GPS® dishes, and either Global HP® or Global Total®. In general, embryos are cultured in Global Total. Global HP is used for thawed zygotes and embryos from patients that are over 35 years of age. We feel that the thawed zygotes benefit from the higher protein and lipid content following thawing. Similarly, we feel that embryos from older patients benefit from the increased factors found in the increased protein content in Global HP. In our laboratory, we do not aliquot culture media into other containers or vessels for storage because we feel the exposure to plastic introduces an additional variable.

15.4.2 Oil Preparation

All oils are washed before use because oil is one of the least defined products used in embryo culture, despite great improvements in manufacturing and testing. Oil quality can be affected by shipping, storage, and light, which are out of the manufacturer's control, and can impact embryo

development [63]. Our center felt that it would be a good practice and would not harm the oil if we washed the oil with culture media that did not contain protein. Therefore, the rationale for washing oil in our center is to remove any potential oil contaminants [64] that might not be detectable with currently used testing methods and to equilibrate the oil with beneficial components found in the media that are also be oil soluble [65].

15.4.3 Preparation of the Culture Dishes

GPS dishes that had been previously off-gassed for at least 48 h are labeled and filled with 11.5–12.5 ml of washed oil. The dishes are allowed to sit for 10–30 min in the laminar hood without the fan on to allow the oil to stabilize in the dish.

The culture medium is placed into a 35 or 60 mm pre-rinsed Nunc® holding dish (depending on the total volume required). The holding dish is rinsed 4–5 times with protein-free HTF medium to remove any potential contaminants that may be present as a result of the dish manufacturing process. Fire-polished, sterile, 9-inch glass Pasteur pipettes are similarly rinsed 4–5 times with HTF. These pipettes are used to underlay the culture droplets with the appropriate Global medium.

Microdrops of approximately 20–30 μl of the medium are used for embryo culture. It is important to note that the oil depth should be at least 1–2 mm over the top of the micro droplets. Culture media can evaporate even in humidified incubators, and culture dishes should be made no later than 1:00–2:00 P.M. on the day prior to the anticipated embryo culture. These steps must be taken to ensure culture media do not evaporate during the culture interval and that the dishes have sufficient time to reach optimum pH prior to use [66].

15.4.4 Incubator Concentrations of CO_2 and O_2

The regulation of pH in the culture medium depends on the bicarbonate concentration in the medium and the CO_2 partial pressure in the incubator. Unfortunately, it is not possible to define an appropriate CO_2 concentration for all circumstances, for at least two reasons. First, for any given CO_2 concentration, the partial pressure decreases with increasing geographical elevation. For example, a 6% CO_2 concentration is required to produce the same CO_2 partial pressure in Denver, as a 5% CO_2 concentration in Boston. Second, measurements of CO_2 concentration are notoriously unreliable. Consequently, the only reliable way to produce the required pH is to adjust the CO_2 concentration until the desired pH is measured in the medium [67]. The manufacturer recommends a pH of 7.27–7.32 for Global Total and Global HP. However, in our experience, embryo development is improved at a pH of approximately 7.23–7.28, and we have increased the CO_2 concentration accordingly.

As noted by Wale and Gardner [68], the atmospheric concentration of oxygen (20%) has been shown to have a wide variety of deleterious effects on the development and function of preimplantation embryos, and this is of particular concern for extended culture to the blastocyst stage [69]. Consequently, we use 5% oxygen for all embryo cultures.

15.4.5 Interrupted and Uninterrupted Embryo Culture

In our laboratory, we use both interrupted and uninterrupted culture. Interrupted culture is used for embryos that are to undergo blastocyst biopsy for preimplantation genetic screening. On Day 3, the embryos are removed from culture, and a small opening is made in the zona pellucida with a laser. The embryos are then moved to fresh dishes for culture through to the blastocyst stage. We move the embryos out of concern for what impact the laser and the elevated temperature might have on the embryo or the culture media. Uninterrupted culture is used for embryos that are not scheduled for biopsy and will undergo a fresh transfer or cryopreservation. These embryos are cultured in the same dish and droplet from Day 1 through to Day 5/6.

It is important to note that we did several sibling trials in our lab before we went to uninterrupted culture. Development to blastocyst was excellent and not different between interrupted (122/200, 61.0%) and uninterrupted (131/220, 59.5%) culture. In addition, we took air samples to ensure the levels of VOCs were as low as possible for our lab. We culture embryos in groups of four to six in some instances but mostly perform single embryo culture so we can be sure of the embryonic history when we are selecting embryos for replacement and/or vitrification.

15.5 Conclusions

- A single medium can support the development of human embryos from the zygote to the blastocyst.
- Interrupted or uninterrupted culture in a single medium from Day 1 onward reduces the possibility of stress associated with changing the medium composition at Day 3.
- Uninterrupted culture may offer some additional advantages over interrupted culture. However, a careful comparison of uninterrupted vs. interrupted culture should be made before introducing uninterrupted culture as general practice in any ART laboratory.

Review Questions

1. What are the advantages of uninterrupted embryo culture?
2. What is the significance of air quality to uninterrupted embryo culture?

3. What precaution should be taken before introducing uninterrupted embryo culture as general practice in the ART laboratory?

4. Are there any precautions that should be taken with plastic materials that come in contact with the embryos while in culture?

5. What precautions should be taken when using oil in embryo culture?

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Sequential Media for Human Blastocyst Culture

David K. Gardner and Michelle Lane

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Learning Objectives

- Benefits of single embryo transfer.
- Negative implications for asynchronous transfer.
- Dynamics of embryo metabolism during the preimplantation period.
- Physiological foundations of sequential media.
- Nutrient gradients exist within the oviduct and uterus.
- Amino acids act as key regulators of development.
- Damaging effects of atmospheric oxygen and ammonium accumulation.
- Blastocyst transfer results in an increase in implantation.
- Sequential media development was among the most rigorously tested procedures in human IVF.

16.1 Introduction

The ultimate goal of human IVF is the birth of a healthy singleton child conceived through the transfer of a single embryo. The benefits that single embryo transfer (SET) brings have been well documented [1]. However, it has taken considerable time and research effort to come close to making this a reality for the majority of patients. The initial success of clinical IVF was limited by a lack of understanding regarding key aspects of embryo physiology resulting in suboptimal culture conditions, impaired embryo development, and all too frequently complete developmental arrest around the eight-cell stage [2–5]. Consequently, it became the paradigm to transfer human embryos to the uterus soon after fertilization on day 1, 2, or 3 resulting in asynchronous development of the embryo within the female tract. Indeed, it was promoted that if the laboratory conditions were not optimal, then embryos should be transferred as soon as possible back to the uterus to avoid the compromised in vitro environment [6]. Ironically, this in vivo environment was also a hostile environment for the cleavage-stage embryo. As a result, implantation rates following the transfer of cleavage-stage embryos to the uterus were very low, and this in turn resulted in the transfer of multiple embryos in order to attain acceptable pregnancy rates. So much so that during the mid-1990s, the average number of embryos transferred in IVF clinics within the USA across all age groups, and oocyte donors, was 4 [7]. With this in mind, in the 1990s, efforts were made worldwide to advance our understanding of the metabolism and physiology of the preimplantation embryo to facilitate improvements in in vitro culture conditions with the goal of increasing the implantation rate of the human embryo, thereby alleviating the need to transfer multiple embryos.

Importantly, in all mammalian species studied to date, including nonhuman primates, the transfer of the cleavage-stage embryo asynchronously to the uterus impairs implantation and fetal development. Rather, in order to ensure high transfer success and good live birth rates, it is important in all mammalian species to transfer the embryo at the blastocyst stage, i.e., replace the embryo in the uterus at the stage in which it resides there [8, 9]. Therefore, we set out to develop a culture system that could support the development of viable human blastocysts at a high rate. Our work led us to analyze the environment of the human embryo in vivo and to determine the effects of medium components on the physiology and metabolism of the preimplantation embryo. This approach led to the development of sequential media, with which it became possible to grow the human embryo to the viable blastocyst stage as a matter of routine. The resultant blastocysts were shown to have significantly higher implantation rates than cleavage-stage embryos and facilitated the elimination of high-order multiple gestations almost overnight and hence helped to pave the way for single embryo transfer while ensuring high pregnancy and live birth rates. Here we describe in detail the journey that led us to formulate sequential media and discuss the potential benefits of blastocyst transfer and how improvements in embryo culture can also impact on embryo selection.

16.2 Physiology of the Preimplantation Human Embryo

If one is to culture any cell type, in order to be successful, it is a prerequisite to understand the physiology of the cell in question. What makes culture of the preimplantation human embryo so interesting (and challenging) is that the physiology of the embryo changes with each successive stage of development. Indeed, the starting point of development, the fertilized oocyte, exhibits a completely different physiology from the blastocyst prior to implantation. The fertilized oocyte and the cleavage-stage embryo are dependent upon pyruvate, lactate, and aspartate to facilitate energy production through low levels of oxidative metabolism [10, 11]. In contrast, the blastocyst has a considerable capacity to utilize glucose as its primary energy source, and does so not only through oxidative metabolism (~50% of the glucose consumed), but also produces considerable lactate through aerobic glycolysis, which is a trait cancers have adopted from blastocysts to support their proliferative and invasive phenotype [12, 13]. The detailed specifics of human embryo metabolism at successive stages of preimplantation development have recently been reviewed elsewhere [14–16]. In order to facilitate the development of a viable preimplantation embryo that will result in the birth of a healthy child, one needs to ensure that the embryo is able to maintain its stage-specific metabolic functions. Aberrations in metabolic function at any stage throughout the preimplantation period not only compromise development prior to transfer but can influence the fetal growth trajectory and in animal models the resultant disease risk of the adult [13, 16, 17]. Hence, analysis of metabolic control during preimplantation embryo development in culture has proven to be an invaluable tool in establishing the suitability of culture media to support normal embryo development and one we have utilized extensively in the development of sequential media [13, 18–21]. Further, with an increased understanding of how metabolic control directly

influences gene expression, through an epigenetic axis [22, 23], the significance of analyzing embryo metabolism has never been more important.

16.3 Development of Sequential Culture Media to Support Development of the Human Blastocyst

Early culture media used in human IVF tended to be rather simplistic, containing just a few salts and carbohydrates. Alternatively, some clinics chose to use tissue culture media, which contained a mixture of nutrients appropriate for somatic cells, some of which are now known to be supraphysiological for embryos, leading to compromised development. The "simple" media, i.e., those lacking amino acids (reviewed in detail in Gardner and Lane [24, 25]) such as those developed initially for the mouse, and adapted for the human, e.g., human tubal fluid medium (HTF) [26], were not effective in supporting human embryos past the eight-cell stage in culture. Indeed, it was not that long ago when it was considered "normal" for the majority of human embryos to arrest around the eight-cell stage in vitro (hence the adoption of cleavage-stage embryo transfers). Consequently, other systems have been tried in attempts to nurture the human embryo to the blastocyst stage in culture. One such system made popular in the 1990s was co-culture, in which human embryos were cultured with oviduct or uterine epithelial cells [27] or specific cell lines [28]. Although the development of blastocysts was facilitated by such technology, co-culture was not widely adopted, likely in part to its complexity and risk profiles associated with cell culture and use of serum. Further, its efficacy was never established in randomized controlled trials. Subsequently, co-culture was replaced with the current approach of the use of more physiological culture media [29].

A key stepping-stone on the path to the development of physiologically based media was the analysis of the in vivo environment of both oviduct and uterine fluids from women at different times during their cycle. Not only were there cycle-dependent differences in the levels of nutrients (as previously determined in an animal model [30]), but crucially there were also significant differences in nutrient concentrations between the oviduct and uterus at the times when the embryo would reside there (Table 16.1) [31]. In essence, the human oviduct is characterized by relatively high levels of pyruvate and lactate and low levels of glucose, while in the uterus, the opposite is true. These findings parallel the known changes in nutrient uptakes and metabolism as the embryo develops and differentiates from a fertilized oocyte to the blastocyst [32–34]. The concentrations of nutrients present in the human oviduct and uterus therefore formed the backbone of the media G1 and G2, the first sequential media designed specifically to support the human embryo.

Evidently, in vivo, the human embryo is exposed to gradients of nutrients as it develops to the blastocyst. The significance of these nutrient gradients is reflected in data from the mouse that show gradients in vitro impact embryo viability following transfer. For example, when the mouse zygote is cultured to the eight-cell stage and then transferred, embryo viability is highest after exposure of the embryo to a high lactate concentration (>20 mM D/L lactate), while when the embryo is cultured post compaction to the blastocyst stage, viability is highest after exposure to lower levels of lactate (<5 mM D/L lactate) [18]. These data support the hypothesis that the physiology of the developing conceptus is temporally regulated by the concentration of nutrients available [35]. Furthermore, the regulation of metabolism of these carboxylic acids changes with development, further highlighting the physiological differences between the zygote and blastocyst stages [16, 35, 36]. The significance of these early observations is further heightened by our more recent understanding that the relative ratio of pyruvate to lactate, which affects the intracellular ratio of NAD^+ to NADH and alters gene expression through ADP ribosylation and acetylation [12, 23]. Hence, the impact of nutrient gradients takes on renewed significance, in that making an embryo adapt to one concentration of nutrients throughout the preimplantation period could feasibly modify its epigenome.

Table 16.1 Concentration (mM) of nutrients in human oviduct and uterine fluids

	Pyruvate	Lactate	Glucose
Oviduct[a]	0.32 ± 0.06*	10.50 ± 1.48*	0.50 ± 0.21**
Uterus	0.10 ± 0.05	5.87 ± 1.19	3.15 ± 0.31

The values of nutrients present in the oviduct and uterus are those present in the media G1 and G2, respectively
Significantly different from uterine values: *, $P < 0.05$; **, $P < 0.01$
[a]Samples taken mid-cycle. Data from Ref [31]

A further major advance in the formulation of embryo culture media came from the determination that amino acids have a significant role to play during embryo development [37–41]. Oviduct and uterine fluids are characterized by high concentrations of the amino acids such as alanine, aspartate, glutamate, glycine, serine, and taurine [42–45]. With the exception of taurine, the amino acids at high concentrations in the oviduct fluid bear a striking homology to those amino acids present in Eagle's nonessential group, i.e., those not required to support somatic cells in culture [46]. Analyses of human and mouse oviduct fluids have also demonstrated that there are significant levels of glutamine present. The functions of such amino acids are listed in Table 16.2. Studies on the embryos of several mammalian species, such as the mouse [40, 47–49], hamster [50–53], sheep [54–56], and cow [19, 57], have all demonstrated that the inclusion of specific amino acids in the culture medium enhances embryo development to the blastocyst stage. In the mouse embryo, it has been determined that inclusion of Eagle's nonessential amino acids and glutamine in the medium significantly increases the rate of zygote development to the blastocyst in

Table 16.2 Role of amino acids during early mammalian embryo development

Role	References
Biosynthetic precursors	[157]
Energy source	[158]
Regulators of energy metabolism	[11, 13]
Osmolytes	[159]
Buffers of pHi	[160]
Antioxidants	[161]
Chelators	[162]
Signaling	[163, 164]
Regulation of differentiation	[59, 165]

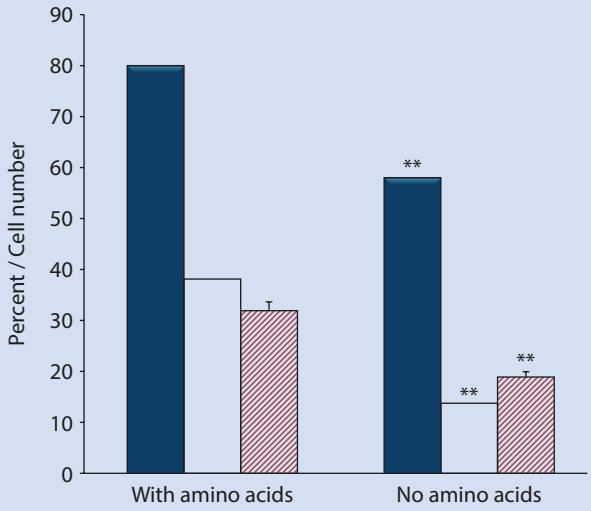

Fig. 16.1 Effect of collection of CF1 mouse zygotes in medium without amino acids on subsequent development. Zygotes were collected in either the medium containing nonessential amino acids and glutamine or the same medium without the amino acids. Embryos were in the collection medium for less than 5 minutes. Solid bars represent morula/blastocyst development. Open bars represent blastocyst development. Shaded bars represent blastocyst cell number. **Significantly reduced compared to collection with amino acids ($P < 0.01$). (Data from Gardner and Lane [48])

culture and indeed can alleviate the two-cell block [48]. Nonessential amino acids and glutamine stimulate cleavage rates [40, 41, 58], blastocyst formation, and hatching of cultured mouse embryos [40, 41, 59]. After compaction, nonessential amino acids and glutamine stimulate cleavage of the trophectoderm and increase blastocoel formation and hatching [59]. In contrast, Eagle's essential amino acids, i.e., those required for somatic cell function, which are at lower concentrations in the oviduct, reduce the cell number of blastocysts from cultured zygotes [40, 59]. Interestingly however, after the eight-cell stage, essential amino acids stimulate cleavage rates and increase the development of the inner cell mass in the blastocyst [59]. Studies on the effects of single amino acids on hamster embryos by Bavister and coworkers [39, 51, 53] found that asparagine, aspartate, glycine, histidine, serine, and taurine stimulated hamster zygote development to the blastocyst in culture, while cysteine, isoleucine, leucine, phenylalanine, threonine, and valine were inhibitory. All the inhibitory amino acids are present in Eagle's essential amino acids, while the stimulatory amino acids to hamster embryo development other than histidine are found in Eagle's nonessential amino acids. A similar stage-specific effect for amino acids was also determined for the embryos of the cow [60].

So, in summary, embryo development in culture is improved in the presence of amino acids, and there exists stage-specific preferences for amino acids. When amino acids are present, more cells are allocated to the inner cell mass of the resultant blastocysts, stage-specific energy metabolism is better supported, there is reduced apoptosis, and most importantly, embryo viability, as assessed by implantation and fetal development, is higher. One could therefore put forward a very strong case that there is no longer a place in clinical IVF for the use of media that lack these key regulators of embryonic function and viability, and that media such as HTF will significantly compromise embryonic developmental potential. Indeed, even a transient exposure of the early embryo to an environment lacking amino acids has downstream consequences for the developing embryo (Fig. 16.1).

When working in vitro, in a system that cannot mimic many of the physical roles of the uterine epithelium, one must be mindful of in vitro artifacts, induced by working outside of the female reproductive tract. For example, amino acids are metabolized by embryos, which subsequently release ammonium [40]. In vivo, one anticipates that this ammonium is subsequently removed by the epithelial cells of the female reproductive tract and then passes through the circulation and is subsequently detoxified by the urea cycle in the liver. However, in a static culture system, i.e., a dish in an incubator, any ammonium produced by embryonic metabolism simply builds up in the medium [40]. To add insult to injury, the amino acids themselves (especially glutamine) are labile at 37 °C and spontaneously deaminate to release ammonium [40]. The significance of spontaneous ammonium buildup is that it not only retards mouse and human blastocyst development in culture [61] but is also associated with subsequent fetal retardation and neural tube defects in mice [41]. Furthermore, it has been shown that ammonium affects embryo metabolism, pHi regulation, and gene expression in both the mouse and human [62–64] and that perturbations induced by ammonium are further compromised by its interaction with atmospheric oxygen (discussed in more detail later) [65]. Interestingly, there appears to be a link between the concentration of ammonium in serum and the induction of fetal oversize in sheep [66, 67].

Even though data are available on the generation of ammonium in the culture medium over time [40, 61, 68, 69], there remains debate concerning the amount of concern one should place on ammonium toxicity in culture medium [70, 71]. Of relevance to this discussion is the reported impact of culture media composition on the live birth rates and subsequent development of the children conceived [72, 73]. In these reports, the effects of two media were analyzed in a day 2 transfer program, and differences in embryo growth kinetics and subsequent birth weight (which persisted through the first 2 years of life) were described. One of the two media employed contained free glutamine, and hence, embryos in this group (which exhibited the growth delay) were exposed to a level of ammonium known to adversely affect human blastocyst development, gene expression, and physiology [63]. As such, it could be hypothesized that the early exposure of embryos to ammonium may explain the effects of the two different media on subsequent birth rate and fetal and neonatal growth trajectory.

The main culprit with regard to amino acid deamination and ammonium release is glutamine. However, this amino acid can be replaced with the dipeptide alanyl-glutamine, which is stable at 37 °C, and its inclusion significantly reduces ammonium release into the culture medium. However, when ammonium production by human blastocysts was quantitated, it was found that the embryo produced significant amounts of ammonium, presumably through amino acid metabolism and transamination [74]. Therefore, it would appear judicious to renew the culture medium used at least every 48 hours in order to circumvent the toxicity of ammonium. From a practical point of view, the amount of work, cost, and embryo manipulations required are the same whether one is working with sequential media or a monophasic system (i.e., one medium formulation for the entire preimplantation period), unless the medium was specifically designed to generate only very low levels of ammonium over extended culture [75]. Even then however, such media cannot account for physiological changes in nutrient gradients, which leads us onto the next section; just how many media do we really need?

16.4 One Medium or Two?

As detailed above, sequential media were developed based on the changing environment within the human female reproductive tract and to accommodate the changes in nutrient requirements, as well as ensuring normal stage-specific metabolism and physiology of the developing embryo [13, 76, 77]. In contrast, the approach of monoculture (one medium for all stages of development) is based on the principle of letting the embryo choose what it wants during development and differentiation. Clearly, such approaches have fundamental differences, and it has been questioned whether the former, more physiological approach, is really required [78]. One such medium is the simplex-derived medium KSOM^{AA}, available for clinical use as Global medium. Simplex medium (SOM) was developed using a computer program to generate successive media formulations based on the response of mouse embryos in culture to form blastocysts [79, 80]. Once a specific medium was formulated, it was tested and blastocyst formation recorded as the sole parameter. There was no assessment of physiology, cell allocation, etc. The program then generated new formulations for use in the next series of cultures. This procedure was performed several times to generate several media that supported high rates of blastocyst development of embryos derived from the oocytes of outbred mice (CF1) crossed with the sperm of an F1 hybrid male [81] and was later empirically modified to KSOM (which contains around 10 times more potassium than SOM) [82]. Such media were subsequently further modified by another laboratory to include amino acids (KSOM^{AA}) [83]. However, this last phase of medium development was based on independent studies on the mouse embryo [40] and did not involve the simplex procedure. This single medium formulation, KSOM^{AA}, was subsequently used to produce human blastocysts in culture [84]. In such types of media, the embryo therefore has to adapt to its surrounding as it develops and differentiates.

We, therefore, decided to perform an analysis of the efficacy of media types on the development of mouse pronucleate oocytes in vitro and to compare their rates of development to in vivo developed embryos [85]. The media chosen were Whitten's (one of the very first mouse embryo culture media) [86], HTF (one of the first simple media developed for human embryos) [26], P1/blastocyst (a glucose- and phosphate-free modification of HTF for 48 h, after which the embryos are transferred to Ham's F10) [87], KSOM (a computer-optimized monophasic medium lacking amino acids) [79], KSOM^{AA} (a computer-optimized monophasic medium with amino acids) [83], and G1/G2 (a sequential media system based on the composition of the human female reproductive tract) [88]. All media were made with the same chemicals and water, and all embryos were transferred to fresh media after 48 h. There was no difference in cleavage time from one- to two-cell stages between any media (Fig. 16.2a). From two- to four-cell stages, embryos cultured in KSOM^{AA}, HTF, and Whitten's media were significantly slower than those in medium G1 (Fig. 16.2b). From four- to eight-cell stages, embryos cultured in all media were significantly slower than in medium G1 (Fig. 16.2c). Rates of compaction were significantly greater in embryos cultured in G1 (74.4%) compared to all other treatments: KSOM^{AA} (60.2%), KSOM (65.9%), P1 (66.0%), HTF (26.4%), and Whitten's (0.0%). Rates of blastocyst development were similar to in vivo developed embryos in media G1/G2 (Fig. 16.3). Blastocyst development in all media was significantly slower than that obtained in vivo and in media G1/G2. Total blastocyst cell number was similar to in vivo blastocysts in media G1/G2, KSOM^{AA}, and KSOM (Fig. 16.4). However, only the ICM development in G1/G2 blastocysts was equivalent to in vivo developed blastocysts (Fig. 16.4). The data obtained demonstrate that embryos cultured in sequential media develop quicker than those in a monophasic system and that

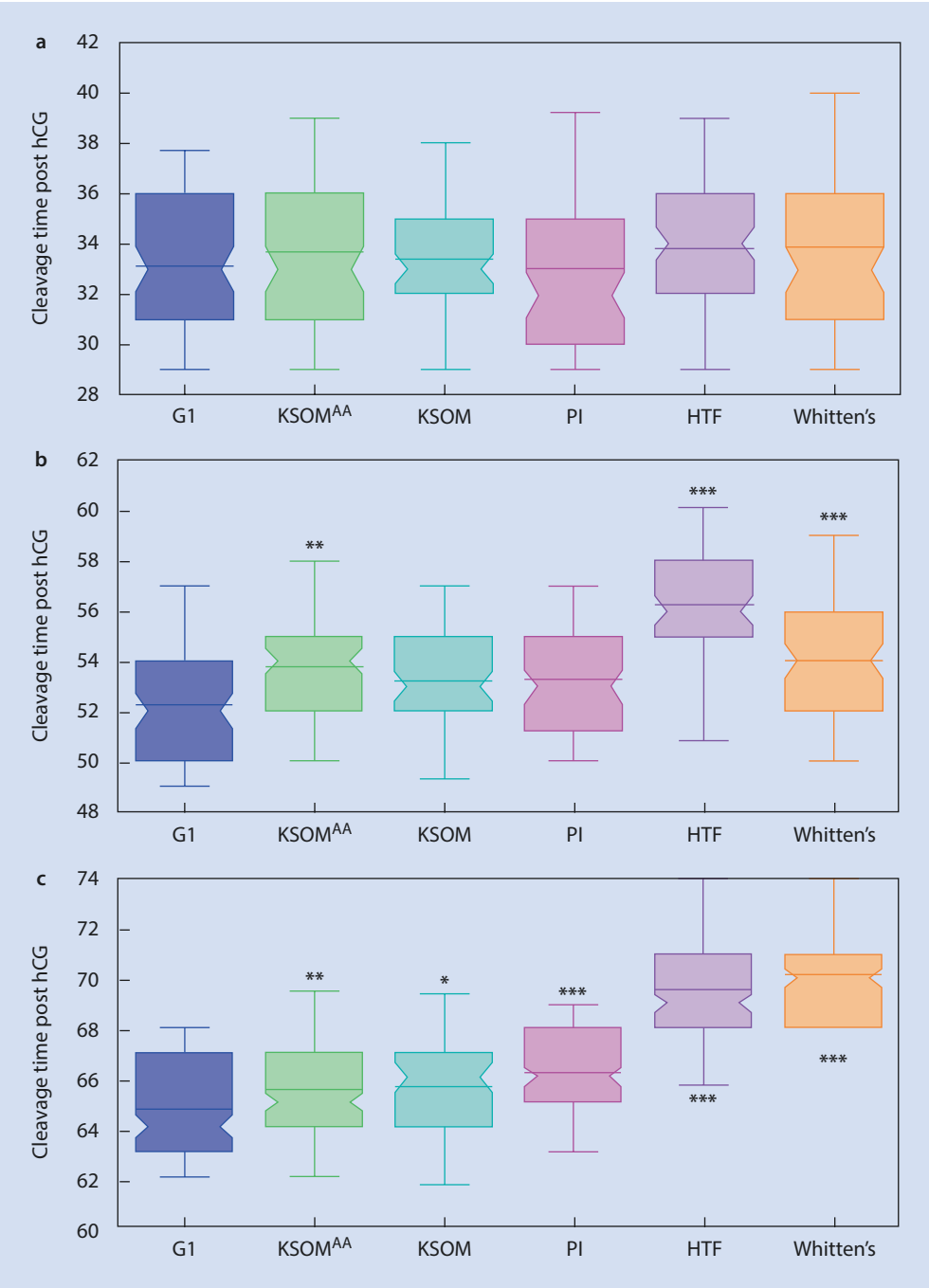

Fig. 16.2 Effect of culture media on cleavage times from the zygote to the eight-cell stage. **a** Cleavage from the one- to two-cell stage. **b** Cleavage from the two- to four-cell stage. **c** Cleavage from the four- to eight-cell stage. Notches represent the interquartile range (50% of the data), and whiskers represent the 5% and 95% quartiles. The line across the box is the mean cleavage time. $n = 100$ embryos per treatment. Significantly different from G1; * $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

the subsequent blastocysts have the same cell number as those embryos developed in vivo. Subsequent transfers also revealed higher implantation rates of mouse embryos cultured in sequential media compared to their siblings cultured in a monophasic medium [77].

Sequential media have now proven themselves to be highly effective in clinical settings [89–91]. However, with the clinical introduction of time-lapse, there has been a resurgence of interest in uninterrupted culture. Therefore, a new single-phase medium (GTL) has been specifically designed to cope with the extended buildup of ammonium from amino acid breakdown and metabolism [75]. While available for use clinically, the literature regarding the efficacy of these media through preclinical and clinical implementation is limited compared to the extensive animal- and human-based literature for other types of media. When consideration is given to the potential metabolic and epigenetic impact nutrient gradients could be having on human embryo physiology, and that physiologically based sequential media have been proven to be highly successful clinically, we still consider sequential media to represent a less stressful and plausibly safer environment for embryo development. Research on the impact of nutrient gradients on the human embryo epigenome is therefore required.

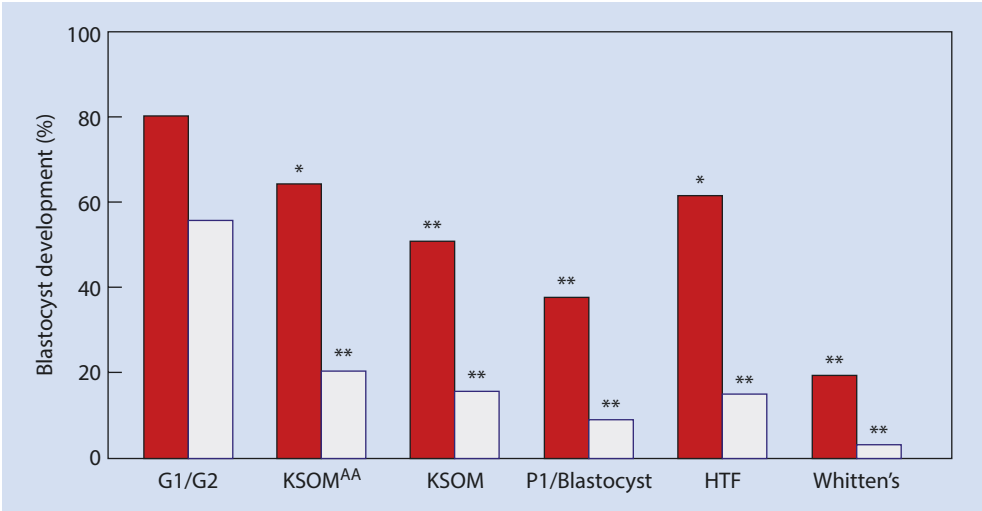

Fig. 16.3 Effect of culture conditions on blastocyst development at 94 h post-hCG. Red bars represent total blastocysts. White bars represent expanded blastocysts. Significantly different from G1/G2 embryos, *$P < 0.05$, **$P < 0.01$. At 94 h post-hCG, 82% of in vivo developed embryos were at the blastocyst stage

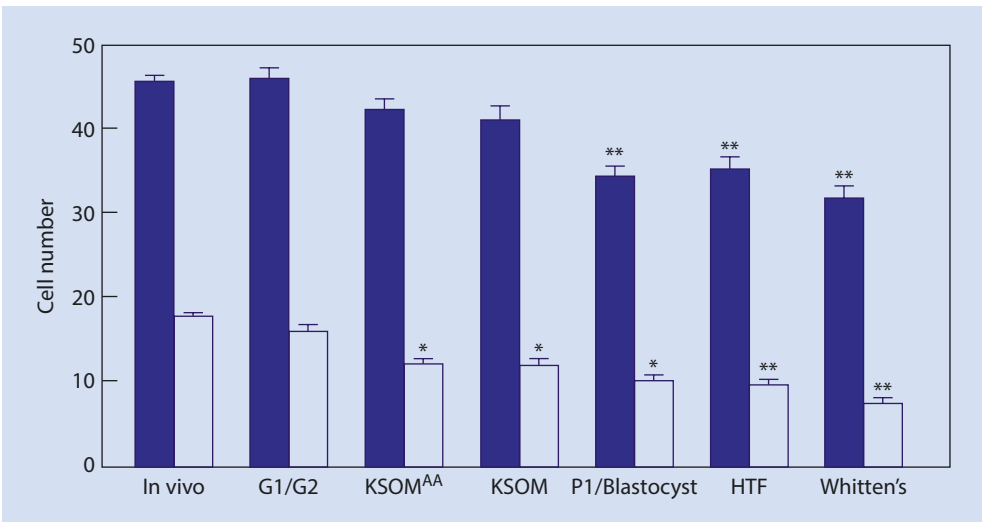

Fig. 16.4 Effect of culture conditions on blastocyst cell number (blue bars) and ICM development (open bars). Significantly different from in vivo, *$P < 0.05$, **$P < 0.01$

16.5 The Holistic Approach to Embryo Culture

It is important to emphasize that the successful culture of viable human embryos entails far more than purchasing the appropriate culture media. Rather, the media is just one aspect of the overall embryo culture system, which itself is just one aspect of the overall laboratory (reviewed in detail; [77]) and that there are likely interactions between media and other aspects of the culture system. Furthermore, it is imperative that sufficient resources be made available for quality control [92] and quality management [93], without which the laboratory is like a plane with no artificial horizon.

One key aspect of the laboratory that is worth reiterating, due to its significant impact on embryo development and viability, is that of oxygen concentration. The fact that both human and F1 mouse embryos can grow at atmospheric oxygen concentration (~20%) has led to some confusion regarding the optimal concentration for embryo culture. The concentration of oxygen in the lumen of the rabbit oviduct is reported to be 2–6% [94, 95], whereas the oxygen concentration in the oviduct of hamster and rhesus monkey is ~8% [96]. Interestingly, the oxygen concentration in the uterus is significantly lower than in the oviduct, ranging from 5% in the hamster and rabbit to 1.5% in the rhesus monkey [96].

Importantly, it has been demonstrated that optimum embryo development of other mammalian species occurs at an oxygen concentration at or below 10% [97–99]. Furthermore, it has been documented that mouse embryos cultured to the blastocyst stage in the presence of 20% oxygen have altered gene expression and perturbed proteome compared to embryos developed in vivo [100–102]. In contrast, culture in 5% oxygen had significantly less effect on both embryonic gene expression and proteome. Further, atmospheric oxygen can affect histone remodeling and methylation patterns in the embryo [103–105]. Recent clinical data in the form of both RCTs and systematic reviews all support the move to the routine culture of human embryos in a reduced oxygen environment [106, 107]. Furthermore, data obtained using time-lapse incubation strongly support the notion that at no time during the preimplantation period should the embryos be exposed to 20% oxygen [108, 109].

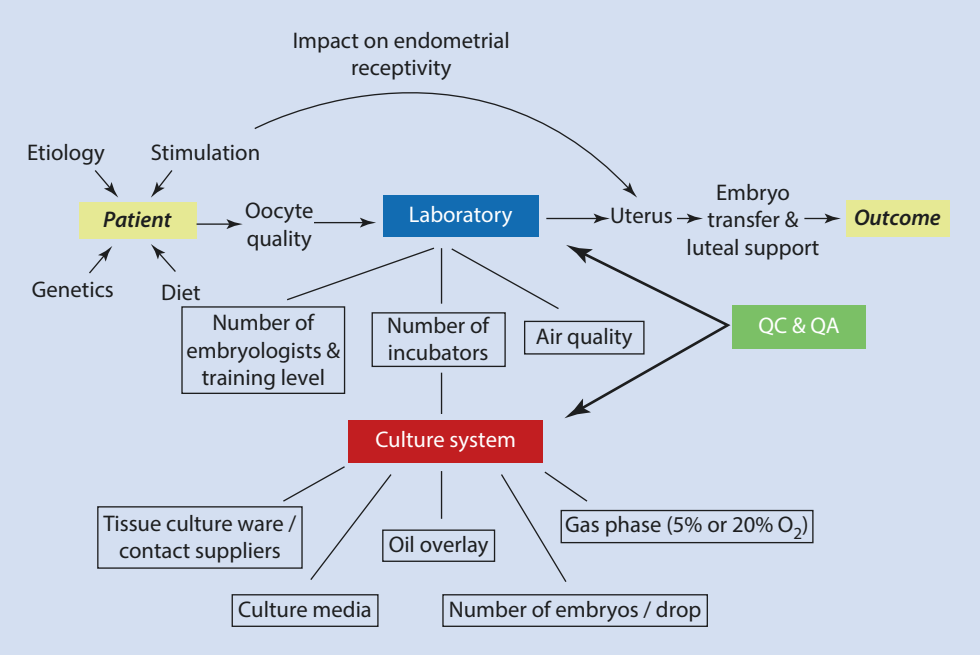

Fig. 16.5 An holistic analysis of human IVF. This figure serves to illustrate the complex and interdependent nature of human IVF treatment. For example, the stimulation regimen used not only impacts oocyte quality (hence embryo physiology and viability [166]) but can also affect subsequent endometrial receptivity [114, 115, 167]. Furthermore, the health and dietary status of the patient can have a profound effect on the subsequent developmental capacity of the oocyte and embryo [168]. The dietary status of patients attending IVF is typically not considered as a compounding variable, but growing data would indicate otherwise. In this schematic, the laboratory has been broken down into its core components, only one of which is the culture system. The culture system has in turn been broken down into its components, only one of which is the culture media. Therefore, it would appear rather simplistic to assume that by changing only one part of the culture system (i.e., culture media), one is going to mimic the results of a given laboratory or clinic. A major determinant of the success of a laboratory and culture system is the level of quality control and quality assurance in place. For example, one should never assume that anything coming into the laboratory that has not been pretested with a relevant bioassay (e.g., mouse embryo assay) is safe merely because a previous lot has performed satisfactorily. Only a small percentage of the contact supplies and tissue culture ware used in IVF comes suitably tested. Therefore, it is essential to assume that everything entering the IVF laboratory without a suitable pretest is embryo toxic until proven otherwise. In our program, the one-cell mouse embryo assay (MEA) is employed to prescreen every lot of tissue culture ware that enters the program, i.e., plastics that are approved for tissue culture. Around 25% of all such material fails the one-cell MEA (in a simple medium lacking protein after the first 24 h) [92]. Therefore, if one does not perform QC to this level, one in four of all contact supplies used clinically could compromise embryo development. In reality, many programs cannot allocate the resources required for this level of QC; when embryo quality is compromised in the laboratory, it is the media that are held responsible, while in fact the tissue culture ware is more often the culprit. (Modified from [77] with permission from Reproductive Healthcare Ltd.)

The holistic approach to looking at embryo culture and transfer is presented in Fig. 16.5.

16.6 Clinical Advantages of Blastocyst Transfer

Some of the inherent advantages of blastocyst culture and transfer are summarized in Box 16.1. For example, not all sperm or oocytes will give rise to a viable embryo [110]. By growing the human embryo beyond the cleavage stages, i.e., past embryonic genome activation [111], one is able to consider the embryo proper, as opposed to an eight-cell embryo, which for the most part can be considered as a cleaving egg. Synchronization of embryonic stage with its relative position in the female reproductive tract is essential for the successful transfer of animal embryos. In all mammalian species studied to date, including nonhuman primates [112], the transfer of cleavage-stage embryos to the uterus (i.e., prior to compaction and therefore before the generation of the first transporting epithelium) results in compromised pregnancy rates compared to the transfer of morulae or blastocysts [9]. Although the human cleavage-stage embryo can develop in the uterus in vivo, the cleavage-stage embryo normally resides in the fallopian tube until day 4 [113]. Consequently, asynchronous transfers are being performed in cleavage-stage IVF cases.

Furthermore, it has been documented in animal models that the environment within the female tract following gonadotropin treatment of the female is not as supportive to embryonic development as a nonstimulated environment [114, 115]. These data question the suitability of the uterine environment following a patient's exposure to exogenous gonadotropins. Clinical data also support the hypothesis that the uterine milieu is compromised following hyperstimulation [116]. Therefore, it may be preferable to expose embryos to such an altered uterine environment for as short a period as possible, which can be achieved through blastocyst trans-

Box 16.1 Advantages of Blastocyst Transfer in Human IVF

- Identification of those embryos with limited, as well as those with the highest, developmental potential through morphological assessment and grading
- Assessment of the embryo post genome activation
- Synchronization of embryo development within the female tract to reduce cellular stress on the embryo
- Minimizing exposure of the embryo to a uterine environment that has been exposed to exogenous gonadotropins
- Fewer uterine contractions by day 5, thereby reducing the chance of embryo being expelled
- Demonstrated higher implantation rates, leading to a reduction in the number of embryos transferred
- Ability to undertake cleavage-stage embryo biopsy without the need for cryopreservation when the biopsied blastomere has to be sent to a different locale for analysis
- Ability to undertake trophectoderm biopsy rather than cleavage-stage biopsy, thereby increasing the number of cells analyzed (typically six to ten) and overcoming ethical/religious concerns of sampling embryonic tissue
- Excellent cryosurvival
- Increase in overall efficiency of IVF

fer. Subsequently, the transfer of human embryos at the blastocyst stage has a sound physiological basis. Several of these advantages were perceived many years ago [27, 28, 117], but it has not been until the last 15 years that culture systems have become practical enough to use as a standard procedure in the IVF laboratory.

16.7 Implementation of Blastocyst Culture

Blastocyst transfer has now been used successfully to treat patients with poor-quality embryos [118, 119], patients with multiple IVF failures [120–122], patients with low numbers of oocytes and embryos [123], and oocyte donors [91]. Although in the examples listed above extended embryo culture has been associated with an increase in IVF success rates, there are a number of reports that question the merits of extended culture [124–126].

Parental factors also affect blastocyst formation and pregnancy-implantation rates. As maternal age increases, both the number and quality of the oocytes retrieved are reduced, thus affecting the outcome of blastocyst transfer [127, 128]. The success of blastocyst culture has also been shown to be under paternal influence [129]. Blastocyst formation is affected by the source of spermatozoa utilized for intracytoplasmic sperm injection, and in close relation to this, clinical pregnancy and implantation rates with blastocyst transfers decrease with the increase in severity of the spermatogenic disorder [130].

Gardner and Balaban [131] reviewed 16 prospective randomized trials using sequential media examining the effect of the day of transfer on IVF cycle outcome [90, 121, 125, 126, 132–143]. Of these 16 trials, 7 reported a significant benefit in the outcome as defined by either increased implantation rate or pregnancy rates (1 study only reported pregnancy rates), when embryos were transferred at the blastocyst stage on day 5 rather than at the cleavage stage. In contrast, only one study found a significant advantage in transferring embryos at the cleavage stage [135]. The remaining eight trials reported no difference in implantation rate with respect to the day of transfer.

The move to blastocyst transfer has had a significant impact on reducing the number of embryos required for transfer, thereby greatly reducing the incidence of high-order multiple gestations. However, although triplets can be confined to the archives of IVF, the overall multiple rate of many programs has not decreased dramatically, as the incidence of twins is typically around 50% when two blastocysts are transferred. The only means to avoid this problem is, therefore, to consider single blastocyst transfer. In the first prospective randomized trial of single versus two blastocyst transfers, in a population of patients with a day 3 FSH $\leq$ 10 mIU/mL and at least 10 follicles >12 mm in diameter on the day of hCG administration (age range 26–43 years), it was possible to establish an ongoing pregnancy rate of 60.9% without any incidence of twins [144]. Significantly, when two blastocysts were transferred, the pregnancy rate rose to 76%, but with a 47.4% incidence of twins.

Therefore, the move to single blastocyst transfer (SBT) appears a more viable alternative to SET on day 3 in good prognosis patients. Papanikolaou and colleagues showed in a prospective randomized trial on SET that not only were higher ongoing pregnancy rates established with blastocyst transfer but that pregnancy loss was lower and birth rate was higher than that obtained with the transfer of cleavage-stage embryos [143, 145, 146].

16.8 Blastocyst Selection for Transfer

As the embryo develops and differentiates into the blastocyst, with its distinct cell types (the inner cell mass and trophectoderm), it becomes feasible to give a more detailed grading to the human embryo. An alphanumeric system [147] has been widely adopted and has proven valuable in assessing blastocyst viability prior to transfer [148–150]. Figure 16.6 shows a human blastocyst that has formed a well-defined ICM surrounded by a cohesive multicellular epithelium, the trophectoderm. Such an embryo from a patient <38 years old, or an oocyte donor, should ideally be transferred alone.

As discussed previously, at the blastocyst stage, one has the ability to examine the embryo proper, and using both proteomic and metabolic analyses, it is evident that even blastocysts with the same morphology have different physiologies [74, 151]. Subsequently, the analysis of embryo metabolism and its secretome will be of great value in quantitating embryonic viability prior to transfer and supporting decisions for single embryo transfer [16].

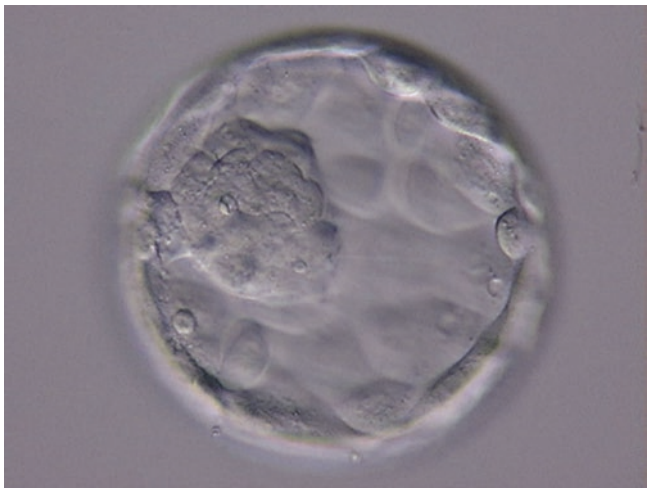

Fig. 16.6 Photomicrograph of a human blastocyst on day 5 of development. Such a blastocyst scores grade 4AA

Box 16.2 Pathway for the Development and Clinical Introduction of the Sequential Media G1/G2

1. Analysis of the nutrient levels within the human oviduct and uterus
2. Use of in vivo developed animal embryos and blastocysts as a "gold standard"
3. Use of several markers of embryo development in vitro including timing of development, gross morphology, blastocyst formation rates, blastocyst cell numbers and cell allocation, and metabolic function (all parameters compared to in vivo controls where possible)
4. Animal embryo transfers (over 5000 embryos transferred in the development of G1/G2)
5. Use of several animal models: hybrid mice, outbred mice, sheep, and cows
6. Preclinical testing on donated human pronucleate oocytes
7. Pilot patient study to ensure safety and efficacy
8. Prospective randomized trial of blastocyst transfer
9. Routine introduction of sequential media in clinical IVF, with monitoring of children born

16.9 Conclusions

With the advent of more physiological culture media, together with better laboratory conditions and management, it is now feasible to culture the human embryo to the blastocyst as a matter of routine. The potential advantages of extended culture go beyond increases in implantation and pregnancy rates and include lower miscarriage rates and the ability to perform comprehensive genetic and physiological analysis of the embryo proper prior to transfer. This in turn has led to a dramatic reduction in the numbers of embryos transferred; we stated earlier that during the mid-1990s, the average number of embryos transferred in IVF clinics within the USA across all age groups, and oocyte donors, was 4. Two decades later, this number has been reduced to 1.7 in patients <35 years old and oocyte donors and to 1.9 for patients <38 years old. In many countries, single blastocyst transfer has become the standard of care [152].

Criticism has been made toward the way in which media for human embryo culture have been developed and introduced [153, 154]. We contend that the sequential media G1 and G2 underwent the most appropriate and extensive development prior to their clinical evaluation and finally their introduction as a routine clinical procedure (Box 16.2). Sequential media have their origins in discovery-based research on the mammalian embryo, including the human, during the early 1990s. Our formulations passed through preclinical phases on multiple animal species, including embryo transfers and live births, extensive trials in the human on donated 2PN embryos, and phase 1 trials and RCTs, and have subsequently been assessed in post-market surveillance by two systematic reviews indicating that optimal blastocyst transfer efficacy is dependent on the use of sequential media.

The capacity to better select a viable embryo will continue to improve the safety of IVF for the mother and child without a compromise in efficacy. The ability of blastocysts to undergo successful cryopreservation with vitrification will culminate in the transfer of genetically analyzed embryos to a naturally cycling uterus. Furthermore, more suitable culture conditions produce embryos more able to survive cryopreservation [155]. The transfer of vitrified blastocysts in a nonstimulated cycle may ultimately be the way all future IVF is performed [156].

Review Questions

1. What are the negative implications of cleavage-stage embryo transfer to the uterus?
2. Can we readily ignore the fact that in vivo the embryos are exposed to nutrient gradients?
3. What is the significance of having amino acids present in human culture media?
4. Does oxygen concentration really matter?
5. How does the detailed development of sequential media compare to that of a monophasic system?
6. What advantages does blastocyst transfer convey?

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In Vitro Maturation of Human Oocytes

Zhi-Yong Yang, Ling Wang, and Ri-Cheng Chian

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Learning Objectives

- Describe the source of immature oocytes derived from.
- Prepare medium for IVM procedure.
- Collect and identify immature oocytes.
- Assess the maturity of immature oocytes.
- Inseminate IVM oocytes and culture fertilized zygotes.
- Determine the timing of embryo transfer for IVM.

17.1 Introduction

Today, pregnancy rates achieved with in vitro fertilization (IVF) treatment have exceeded that achieved in natural cycles [1–3]. These figures are achieved with simultaneous transfer of multiple embryos in a given treatment cycle. Traditionally, production of multiple embryos has been only possible after development of controlled ovarian stimulation (COS) protocols.

In a natural menstrual cycle, several antral follicles are present in the human ovary. The interactions between the growing follicles, the pituitary gland, and the hypothalamus prevent multifollicular growth and allow only one follicle complete maturation and reach ovulation in the majority of cycles. COS with exogenous gonadotropins and gonadotropin-releasing releasing hormone analogs enables overriding this natural selection process and collection of multiple mature oocytes, which are amenable to fertilization. COS has been an integral part of conventional IVF treatment for over 20 years.

COS requires multiple daily injections and frequent monitoring scans creating direct and indirect costs, loss of working time, and inconvenience. Unfortunately, the cost of drugs used for COS poses a substantial financial burden and at times prevents a couple's access to treatment. The most important medical problem associated with COS is the risk of ovarian hyperstimulation syndrome (OHSS). OHSS is a potentially lethal condition, most commonly occurring as an iatrogenic complication of COS [4]. It is characterized by ovarian enlargement and increased capillary permeability, causing fluid shift to the third space. This results in ascites formation, hypovolemia, hemoconcentration, and hypercoagulability. OHSS may be further complicated by acute renal failure, hypovolemic shock, thromboembolic episodes, and adult respiratory distress syndrome (ARDS), and in extreme cases, it can be fatal. The risk of OHSS can be as high as 6% in young women with polycystic ovarian syndrome (PCOS) [5]. More recently, the altered hormonal milieu during COS has been suggested to have detrimental effects on developing oocytes, embryos derived from these oocytes, and/or endometrial receptivity [6, 7].

Collection and in vitro maturation (IVM) of the already existing immature oocytes in the smaller antral follicles can provide multiple mature oocytes that can be fertilized or cryopreserved for future use. The technique has been named IVM. Using immature oocytes collected in a nonstimulated cycle to produce multiple embryos avoids the costs, inconvenience, and risks associated with conventional IVF.

Research on immature oocytes dates as far back as the 1930s [8]. These oocytes are shown to resume meiosis upon removal from the follicle, have the capacity to complete meiotic division, and can be fertilized in vitro. More than 80% of oocytes were reported to resume meiosis independent of the menstrual cycle day and gonadotropin support in IVM medium [9]. The first pregnancy and live birth from in vitro matured oocytes in humans were only reported by Veeck and colleagues in 1983 in the context of a stimulated IVF cycle [10]. They reported two pregnancies resulting from IVM of immature oocytes collected alongside mature oocytes in gonadotropin-stimulated IVF cycles. However, IVF with COS had already become the norm, and it was not until 1991 when Cha and colleagues first reported intentional collection of immature oocytes from women undergoing gynecological surgery that IVM began to gain momentum [11]. The immature oocytes donated by these women were matured in vitro, and the resulting embryos were transferred to a recipient with premature ovarian failure [11]. The recipient delivered healthy triplet girls.

Three years later, Trounson and colleagues reported the collection of immature oocytes from women with PCOS for their own use [12]. The immature oocytes collected were matured in vitro with gonadotropin-enriched medium, then fertilized, and a healthy live birth following transfer of resultant embryos was reported. Unfortunately, the initial pregnancy rates were low, and it took another 5 years to allow success rates to exceed 35% per cycle in appropriately selected patient groups [13–24].

17.2 Indications for IVM Treatment

17.2.1 Infertility with PCO or PCOS

Young women with high antral-follicle counts achieve the highest pregnancy rates with IVM [25]. IVM has become an established treatment option for women with PCO or PCOS who need ART. However, the clinical application of IVM technology can be expanded to benefit other patient populations.

17.2.2 High Responders to Gonadotropin Stimulation

IVM combined with IVF can be a valid option for patients who demonstrate an overresponse to COS and are considered to be at risk of OHSS during an already started IVF cycle. The only method proven to prevent OHSS completely is to avoid the injection of human chorionic gonadotropin (HCG) for final oocyte maturation [4]. However, this strategy requires cancelation of the treatment cycle, leading to frustration in both the patient and the physician. Giving the HCG injection when the leading follicle size is 12–14 mm, before the conventional HCG criteria for IVF is met, followed by oocyte collection 36–38 h later may prove to be an effective strategy. Lim et al. reported a 36.6% clinical pregnancy rate using a similar strategy in 123 women who had

≥20 follicles with a mean diameter ≥ 10 mm after ≥5 days of gonadotropin stimulation [26]. None of the women in this cohort developed OHSS. As a further note, 18.9% of 1554 oocytes collected were in vivo matured.

17.2.3 Poor Responders to Gonadotropin Stimulation

Unfortunately, all women do not respond similarly to COS, and some fail to develop a reasonable number of mature follicles in stimulated IVF cycles. The most common cause of poor response seems to be the age-related decline in ovarian reserve, but it also occurs in some younger women.

For women in whom poor ovarian response does not seem to be due to a rectifiable cause inherent to the particular treatment cycle, i.e., inappropriate choice of stimulation protocol, skipped medication, etc., trying further stimulated cycles can prove useless. IVM may provide a viable option in such cases. In two studies involving women with a history of poor ovarian response in a stimulated IVF cycle, pregnancy rates of 31.6 and 40.4% were achieved with IVM [27, 28]. In eight women with a poor response, defined as ≤4 follicles growing or oocytes collected in a previous stimulated IVF cycle, we achieved a similar number of embryos available for transfer in the subsequent IVM cycle [29]. Six women reached embryo transfer (75%), and one achieved a live birth, yielding a 16.7% live birth rate per transfer in this small sample.

17.2.4 Oocyte Donation

Young women who have high ovarian reserve are preferred as oocyte donors. Unfortunately, such women comprise a high risk population for early OHSS in stimulated IVF cycles. Besides the risk of OHSS, the inconvenience of the numerous injections required and the theoretical risk of cancer associated with repeated use of ovulation induction drugs cause reluctance on the part of some potential oocyte donors. IVM can be an appropriate method for oocyte donation cycles as young women with high antral-follicle counts comprise the best candidates for IVM and yield good pregnancy rates.

We reported collection of an average of 12.8 immature oocytes from 12 oocyte donors with a mean age of 29 years. Sixty-eight percent of the oocytes matured in vitro and 62 embryos were available for transfer to 12 recipients with a mean age of 37.7 years [30]. On average, four embryos were transferred (range 2–6), and a clinical pregnancy rate of 50% was achieved. Two women had first-trimester miscarriages while four had healthy live births, yielding a live birth rate of 33%.

17.2.5 Fertility Preservation

The American Society of Clinical Oncology and American Society of Reproductive Medicine have endorsed IVF and embryo cryopreservation (EC) as the only method of female fertility preservation [31, 32]. IVM expands the fertility preservation options for women who are not candidates for IVF–EC for various medical and social reasons. Women with hormone-sensitive tumors may undergo immature oocyte collection and cryopreserve resultant embryos. The advantages of IVM are not limited to eliminating the need for expensive drugs as well as their administration and avoiding hormone-sensitive tumors: IVM enables oocyte retrieval at any phase of the menstrual cycle and completion of the fertility preservation procedure in 2–10 days, preventing a delay in treatment of the primary disease [33, 34]. We reported three women without male partners seeking fertility preservation prior to chemotherapy, who presented for the first time in the luteal phase of their menstrual cycle and were to undergo gonadotoxic treatment immediately [34]. Five to seven immature oocytes were recovered with luteal-phase oocyte retrieval from these patients. Three to five M-II oocytes were vitrified following IVM. Two of these three women later underwent one and two more collections, respectively, in the follicular phase of the next cycle(s), and additional immature oocytes were vitrified following IVM.

Immature oocytes can also be harvested from ovarian biopsy specimens and can be vitrified following IVM [35]. This combination of ovarian-tissue cryobanking and IVM represents a new strategy for fertility preservation. We retrieved 11 immature oocytes from a wedge resection specimen in a 16-year-old patient with mosaic Turner syndrome. Eight of these oocytes were vitrified following IVM [36]. In four women with cancer, we harvested 11 immature and eight mature oocytes from wedge biopsy specimens. Eight of the 11 immature oocytes reached M-II stage following IVM and were vitrified [35].

17.3 Step-by-Step Protocol for IVM Treatment

17.3.1 Monitoring and Management of an IVM Cycle

Monitoring starts with a baseline scan performed in the early follicular phase of the menstrual cycle, preferably between days 2 and 5 of a natural menstrual cycle or a withdrawal bleed, induced with progesterone administration in amenorrheic women. The number and size of the antral follicles and endometrial texture and thickness are recorded. The uterus and ovaries are examined for any abnormalities. A second scan is performed about a week later when it is anticipated that the largest follicle has reached 10–12 mm in diameter and the endometrial thickness is at least 6 mm. The presence of a dominant follicle does not require cancelation of the treatment cycle because smaller follicles are found to contain viable oocytes, even in the presence of a dominant follicle [37, 38].

The role of gonadotropin administration before oocyte collection is still controversial. Randomized controlled trials comparing the outcome of gonadotropin-primed IVM cycles with that of IVM cycles without any priming have yielded

conflicting results [20, 22, 39, 40]. When data from these studies are combined, there is a trend toward higher clinical pregnancy rates with FSH/HMG administration or HCG priming in women with polycystic ovaries (PCO)/PCOS; however, the difference is not statistically significant. Differences in patient characteristics, gonadotropin administration protocols, and the limited total number of patients included in these studies prevent a definitive conclusion, and further research is needed. Based on our own experience and the favorable trend observed in trials of HCG priming, the current routine IVM protocol at the McGill Reproductive Centre (MRC) involves HCG administration priming regardless of cycle regularity.

Our preferred HCG dose is 10,000 IU i.m. 36–38 h before oocyte collection. The decision on dosage is based on failure to observe any improvement in laboratory or clinical outcomes with a higher HCG dose of 20,000 IU in a randomized controlled trial conducted in our unit [41]. On the other hand, in a retrospective analysis, we found an increase in the number of in vivo matured oocytes collected, the rate of oocyte maturation in the first 24 h after collection, and the embryo implantation and clinical pregnancy rates when the interval between HCG administration and oocyte collection was extended to 38 h rather than the traditional 35 h [42]. The relevance of the latter findings is supported by the following studies. Our results suggest that the presence of in vivo matured oocytes can be associated with higher pregnancy rates in HCG-primed IVM cycles [43]. A significantly higher proportion of embryos derived from in vivo matured oocytes attained good morphological characteristics compared with those derived from in vitro matured oocytes in these cycles. Moreover, oocytes that complete IVM in the first 24 h after collection seem to have a higher rate of cleavage, and embryos derived from such oocytes have a higher rate of blastocyst formation compared with their counterparts that complete maturation later [44].

Both the size of the leading follicle and endometrial thickness are taken into consideration regarding the timing of oocyte retrieval. We prefer the size of the dominant follicle to range between 7 and 12 mm at the day of HCG administration, i.e., 36–38 h before oocyte collection, because embryo implantation and clinical pregnancy rates were found to be significantly lower in cycles where the mean diameter of the dominant follicle measured >14 mm compared with cycles that produced smaller dominant follicles with a mean diameter of 10–14 mm or ≤10 mm at the time of oocyte collection [37]. The highest implantation (14.3%) and pregnancy rates (40.3% per transfer) were achieved in cycles with a 10–14-mm-sized dominant follicle.

In a retrospective analysis of 155 unstimulated IVM cycles, we have found mean endometrial thickness to be significantly higher in conception cycles, though the absolute difference was only 0.8 mm (10.2 vs. 9.4 mm in conception and nonconception cycles, respectively, $p = 0.04$). More interestingly, a trend analysis demonstrated a significant increase in pregnancy rates in parallel with endometrial thickness (clinical pregnancy rates were 9.4, 15.9, 27.6, and 28% for endometrial thickness of <8, 8–9.9, 10–11.9, and ≥12 mm, respectively; chi square test for trend, $p = 0.036$) [45]. Interestingly, endometrial texture, categorized as "triple line" or "non-triple line," was not found to be predictive for treatment outcome in the same study.

If the endometrial thickness is <6 mm on the day of the second scan, HCG administration can be delayed if the size of the largest follicle is less than 12 mm. Sometimes, if the largest follicle is close to 10–12 mm but the endometrium is very thin, we would use estradiol alone in a dose of 12 mg/day. This increases the endometrial thickness and delays follicle growth.

For women whose endometrial thickness was less than 6 mm on the day of the second scan, we later adopted an additional strategy of administering HMG in a dose of 150 IU/day and/or starting estradiol earlier. The rationale is increasing endogenous estrogen levels with limited support to the continuing growth of the follicles in the cohort. A retrospective comparison of the two strategies in 48 cycles demonstrated a similar increase in mean endometrial thickness with both methods, from 4.5 to 7.7 mm and from 4.7 to 7.3 mm in HMG and estradiol cycles, respectively [46]. However, compared with oral administration of 17β estradiol, HMG injections were associated with an increase in the number of in vivo matured oocytes collected (cycles with more than one in vivo matured oocyte, 54.5 vs. 34.6%), implantation (15 vs. 8.2%), and pregnancy rates (36.4 vs. 23.1%). The differences were not statistically significant, arguably due to the limited sample size of the study. Currently, we prefer HMG over estradiol for women with a thin endometrium. HMG injections are continued until endometrial thickness reaches 8 mm or the leading follicle reaches a mean diameter of 12 mm.

17.3.2 Oocyte Collection Procedure

The principles of transvaginal ultrasound-guided oocyte retrieval for IVM are the same as those for IVF oocyte collection. There are a few modifications in both the technique and equipment. Most patients easily tolerate the procedure under conscious sedation and with paracervical block achieved with 1% bupivacaine injection. A smaller-gauge needle (19–20G) with a shorter bevel is used. The aspiration pressure is set at 75–80 mmHg, approximately half the conventional IVF aspiration pressure, in order to minimize the risk of oocyte denudation during aspiration. This precaution is carried out because immature oocytes need the presence of surrounding granulosa cells during the nuclear maturation process. The fine-bore needle may be blocked frequently with blood-stained aspirate and ovarian stroma. Therefore, multiple punctures are often needed, and flushing the needle lumen with heparinized saline between punctures is required. Sometimes external abdominal pressure may be required to fix the mobile ovaries during collection. Patients with difficult-to-reach ovaries or poor pain control may do better under limited general anesthesia with propofol.

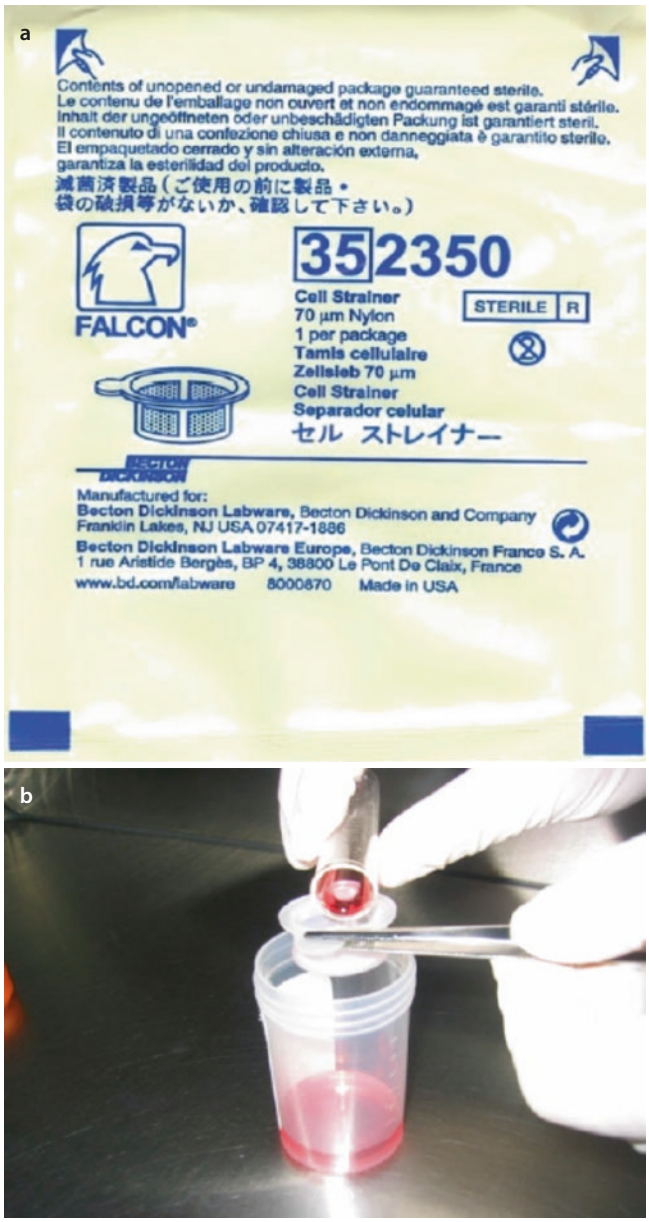

Fig. 17.1 **a** Labeling on Cell Strainer (Falcon, Becton Dickinson and Company, USA); **b** follicular aspirates are filtered with a Cell Strainer

17.3.3 Laboratory Procedure

Similar to IVF, the follicular aspirate is first examined under a stereomicroscope to identify cumulus-oocyte complexes (COCs). Since identification of small immature oocytes surrounded by scarce granulosa cells, which are not widely dispersed, is more difficult with the conventional technique as they can be overlooked, follicular aspirate is filtered through a nylon mesh strainer (Fig. 17.1) with 70 µm pores after the removal of initially identified COCs. The filtered aspirate can be reexamined after washing with HEPES-buffered human serum albumin (HSA)-containing medium.

In order to determine whether the oocyte is mature or not, a special observation technique called "sliding method" can be employed without denuding cumulus cells from the oocyte.

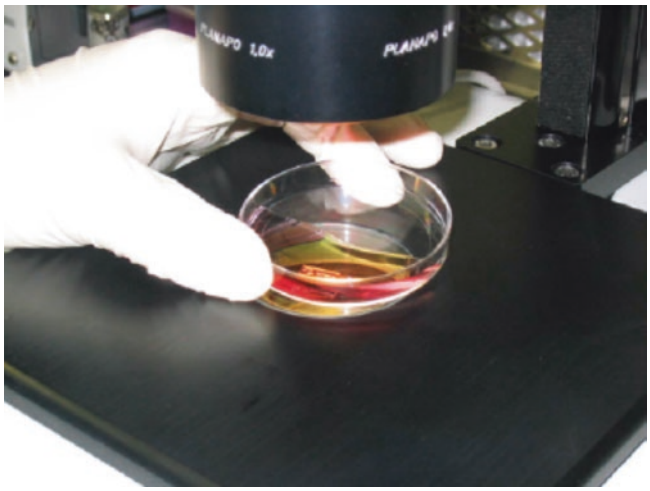

Fig. 17.2 Sliding technique for observation of oocyte maturity. COCs are allowed to slide slowly from one side to the other on the bottom of the Petri dish, while being observed under the stereomicroscope

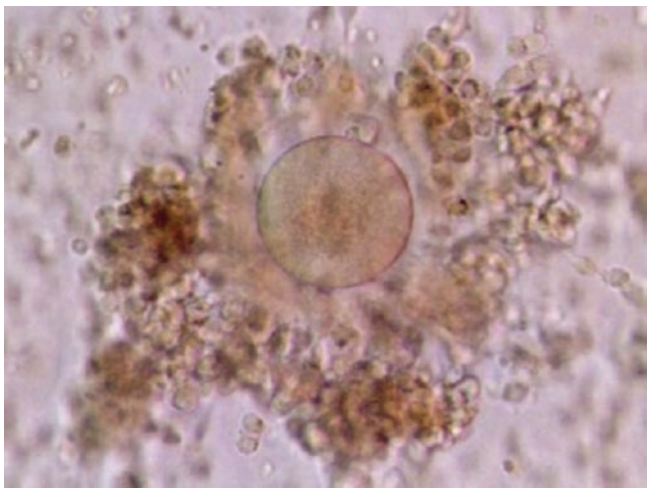

Fig. 17.3 During COC sliding, it is possible to observe clearly whether or not the oocyte has extruded a first polar body (1PB) into perivitelline space (PVS)

Briefly, COC is allowed to slide slowly from one side to the other on the bottom of the Petri dish, while being observed under the stereomicroscope (Fig. 17.2). During COC sliding, it is possible to observe clearly whether or not the oocyte cytoplasm contains a germinal vesicle (GV) or if the oocyte has extruded a first polar body (1PB) into perivitelline space (PVS) (Fig. 17.3). If neither GV is seen in the oocyte cytoplasm nor 1PB found in PVS, the oocyte is defined as germinal vesicle breakdown (GVBD) or metaphase-I (M-I) stage.

The immature COCs (maximum of ten) are incubated in an Organ Tissue Culture Dish (Falcon, 60 × 15 mm) (Fig. 17.4) containing 1 mL oocyte maturation medium supplemented with a final concentration of 75 mIU/mL FSH and 75 mIU/mL LH at 37 °C in an incubator with an atmosphere of 5% CO_2 and 95% air with high humidity (prefer with triple gas mixture, 90% N_2, 5% CO_2, and 5% O_2 with 100% humidity). Oocyte maturation medium should be prepared

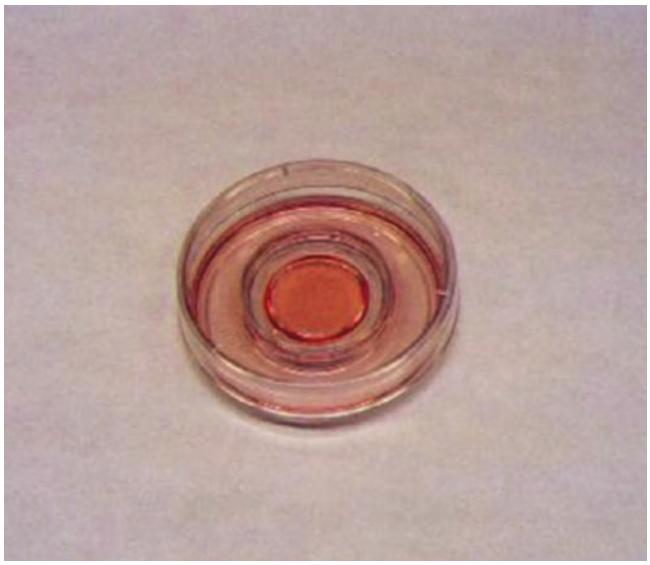

Fig. 17.4 Organ Tissue Culture Dish (Falcon, 60 × 15 mm) containing 1 mL oocyte maturation medium in the inner well and 2 mL of oocyte maturation medium in the outer well

for equilibration at least 2 h before immature oocyte retrieval (particularly, it can be made 1 day before). Some commercially available oocyte maturation media do not contain a protein source. In that case, the oocyte maturation medium needs to be supplemented with some protein source, such as HSA or maternal serum. If maternal serum is going to be used, it is better prepared in advance and inactivated (56 °C 30 min) before using.

Oocytes reaching metaphase-II (M-II) stage on the day of collection are denuded and fertilized together with any in vivo matured oocytes, while immature oocytes are cultured in IVM medium for 24–48 h. Twenty-four hours after maturation in culture (Fig. 17.5), all COCs are stripped for identification of oocyte maturity. COCs are denuded using a finely drawn glass pipette following 1 min of exposure to a commercially available hyaluronidase solution. The mature oocytes are then subjected to insemination by either IVF or intracytoplasmic sperm injection (ICSI) after stripping. The remaining immature oocytes (GV and M-I) will remain in culture for another 24 h. Forty-eight hours after oocyte retrieval, the remaining stripped oocytes are reexamined, and if any have matured at this point, they will be inseminated by either IVF or ICSI.

Since in vivo matured oocytes can be retrieved after HCG priming from leading or dominant follicles, it has been argued that such "IVM treatment" is not genuine. Accordingly, the treatment has been reworded as "natural cycle IVF combined with IVM," simply referred to as natural cycle IVF/M [38, 47–49].

Although similar implantation and pregnancy rates have been reported following fertilization of in vitro matured oocytes with ICSI or IVF, ICSI has been commonly practiced in IVM cycles due to a theoretical risk of zonal hardening during the in vitro culture period [50, 51]. In fact, fertilization rates with ICSI were shown to be higher than with IVF

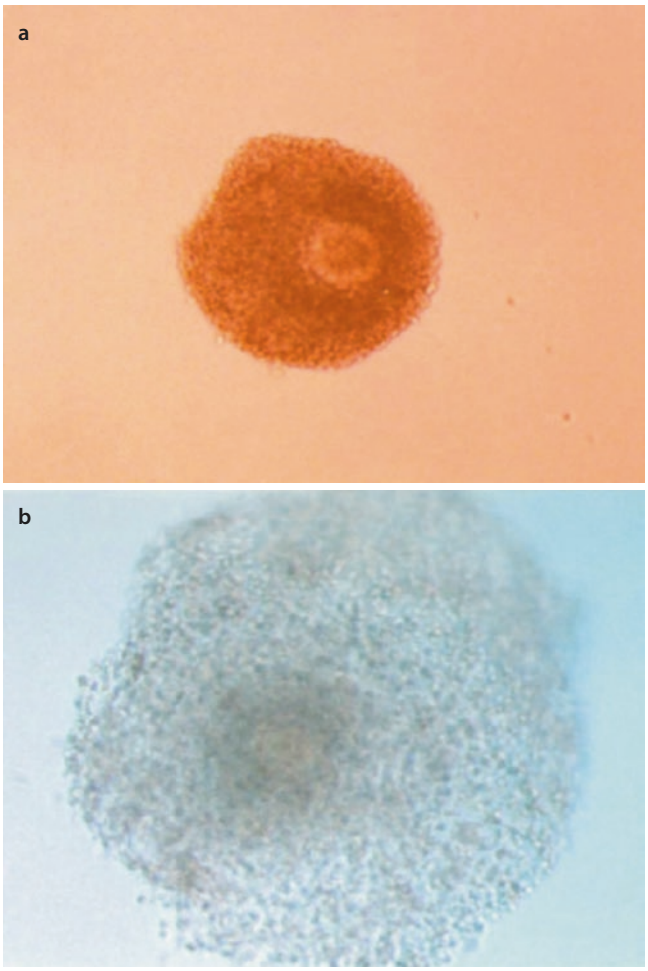

Fig. 17.5 Morphological changes of immature COC before and after culture in the oocyte maturation medium. **a** Immature COC immediately after retrieval from the ovary; **b** the same COC after 24 h of culture in the oocyte maturation medium. Note that the cumulus mass has almost doubled in size compared with its mass before culture (two photos with the same magnification)

in the same study (84.1 vs. 56.3%) [51]. Another reason for preferring ICSI over IVF in IVM cycles is some immature oocytes being denuded for the assessment of polar-body extrusion. Oocytes devoid of cumulus cells may have decreased chemotactic potential for sperm in the medium [52]. ICSI is the fertilization method routinely used in the McGill IVM program. The preferred time for ICSI is 2–4 h after polar-body extrusion [52].

Culture conditions for fertilized oocytes and cleavage-stage embryos derived from in vitro matured oocytes are the same as those in IVF cycles. Embryo development and quality are similarly assessed, based on the number of blastomeres and the amount of nuclear fragments.

17.3.4 Embryo Transfer

The timing of embryo transfer and the number of embryos to be transferred are dictated by the number and quality of available embryos. Embryo transfer is commonly performed on

the third day after oocyte collection. Growth and quality of available embryos are evaluated with regard to fertilization time of each embryo. A group of best embryos for days is transferred using essentially the same technique as that employed in IVF cycles. In general, embryo implantation rates in IVM cycles are lower than in IVF cycles. Therefore, on average, more embryos are transferred in order to maintain similar pregnancy rates. However, as expected, this fact does not seem to increase multiple pregnancy rates after IVM [53, 54].

High implantation and pregnancy rates have been achieved by performing blastocyst transfers in selected IVM patients [55]. A clinical pregnancy rate of 51.9% and an implantation rate of 26.8% have been reported with blastocyst transfer in patients with >7 zygotes and >3 good-quality embryos on the third day postfertilization in IVM cycles [55]. Assisted hatching is routinely employed before embryo transfer in our IVM program due to the abovementioned concerns about zonal hardening [50].

17.3.5 Luteal-Phase Support

The luteal-phase support protocol employed in our IVM program includes 50 mg/day i.m. progesterone injections and 6 mg/day estradiol valerate p.o. in three divided doses. We continue luteal-phase support until completion of the first trimester for pregnant patients.

17.4 Clinical Outcome of IVM Treatment

The most important determinants of pregnancy following an IVM cycle are the female age and the number of oocytes collected. Young women with PCO seem to be the best candidates for IVM treatment. In 2007, we achieved an embryo implantation rate of 15% and a clinical pregnancy rate per embryo transfer (CPR) of 36.7% in women younger than 35 years of age. However, for women aged between 35 and 40 years, implantation and CPR were 10.1 and 29.3%, respectively, in the same period. Similarly, successful results have been reported by different centers around the world [14]. Pregnancy rates seem to be significantly higher when an in vivo matured oocyte has been collected. Recently, we reported a 40% clinical pregnancy rate in such cycles in young women with PCO [43].

Immature oocytes priming with FSH or HCG prior to immature oocyte retrieval improves oocyte maturation rate and embryo quality as well as pregnancy rates when retrieved from infertile women with PCOS and normal ovaries and regular menstrual cycling women. The source of follicles may be important for the subsequent embryonic development, but the developmental competence of immature oocytes derived from the small antral follicles seems to not be adversely affected by the presence of a dominant follicle, which developed a newly named natural cycle IVF combined with IVM, because there will be in vivo matured oocytes and immature oocytes retrieved at the same time and in the same cycle. So far, it has been estimated that there are more than a few thousands of healthy infants born following immature oocyte retrieval and IVM from women with PCOS [56, 57]. In general, the clinical pregnancy and implantation rates per embryo transfer have reached approximately 35–40% and 15–20%, respectively, in infertile women with PCOS following IVM of immature oocytes based upon different reports [56]. If it included the mentioned natural cycle IVF combined with IVM, the clinical outcomes must be higher than those numbers.

Since the introduction of IVF and other assisted reproductive technologies for infertility treatment, the health of infants born from these techniques has been a major concern, including IVM technology, especially for the extra period of culture of immature oocytes in vitro. In fact, IVM of the immature oocytes from GV via M-I to M-II stage normally require 36–48 h of culture in vitro [9, 13]. For clinical IVM of the immature oocytes from M-I to M-II stage, it only needs 6–24 h of culture in vitro [58]. Interestingly, it has been reported that there was no significance in morpho-kinetics of embryos developed from oocytes matured in vivo and in vitro in IVM cycles, indicating that the embryonic developmental potential may not be different between the oocyte matured in vivo and in vitro [59]. There were insignificant differences for zona pellucida birefringence and meiotic spindle between the in vivo and in vitro matured oocytes [60]. Furthermore, it has been indicated that the in vivo and in vitro matured oocytes showed normal ooplasm showing uniform distribution of organelles [61]. Mitochondrial morphology appeared similar between the maturation conditions. Cortical granules were found typically stratified in a single, mostly continuous row just beneath the ooplasm in in vivo and in vitro matured oocytes.

There was a study showing that optimized human IVM procedures have no significant effects on the establishment of maternal DNA methylation patterns at LIT1, SNRPN, PEG3, and GTL2 [62]. There were no differences in terms of oxygen consumption between embryos derived from the in vitro and in vivo matured oocytes, indicating that also there was no imprinting gene disorder founded from IVM babies [63]. No statistically significant impact was found of IVM on chorionic villus and cord-blood DNA methylation at the studied developmentally important genes and interspersed repeats, suggesting that IVM-induced epigenetic changes in offspring, if any, will be relatively small in magnitude and/or infrequent [64]. For human IVM, it seems no definitive conclusion on imprinting establishment can be drawn as well-designed studies are currently not available. Therefore, epigenetic analysis should be performed in children born from pregnancies after IVM to draw definitive conclusions on the epigenetic safety of human IVM [65].

The number of live births from IVM oocytes has been increasing over the past three decades. It has been estimated that more than 5000 IVM infants have been born worldwide [56]. There are many concerns about IVM infants as to obstetric and perinatal outcomes as well as long-term development [66]. Several studies have reported that the mean birth weight

and the incidence of congenital anomalies seem to be comparable with spontaneous conceptions or conceptions of infertile women undergoing IVF treatment [54, 67, 68]. It was reported that 196 infants conceived after IVM of immature oocytes were not associated with an increased risk of adverse obstetric or perinatal outcomes compared with children conceived by in vivo matured oocytes or children conceived by conventional ovarian-stimulated ICSI cycles [69].

17.5 Pregnancy Loss

Biochemical pregnancy loss rates were similar among 1581 women who had a positive pregnancy test following assisted reproduction treatment (ART) with IVM, IVF, or ICSI in our unit during a 5-year period (17.5% for IVM pregnancies, 17% for IVF, and 18% for ICSI pregnancies, $p = 0.08$). However, the clinical miscarriage rate was significantly higher in IVM pregnancies (25.3%) than in IVF (15.7%) and ICSI (12.6%) pregnancies ($p < 0.01$) [70]. However, the incidence of PCOS in the IVM group was 80%, whereas only 8% and <1% of women in the IVF and ICSI groups had PCOS, respectively. Miscarriage rates reaching 25% after ovulation induction, and ranging from 25% to 37% following IVF, have been reported in women with PCOS [71–73]. Arguably, the higher incidence of PCOS in IVM patients in this series can be the reason for the higher miscarriage rates observed in the IVM group. Critically, miscarriage rates were not different between IVM and IVF pregnancies among women with PCOS, 24.5 vs. 22.8%, respectively.

17.6 Obstetric Outcome and Congenital Abnormalities

IVM pregnancies comprised 15.9% of 344 ART pregnancies in a retrospective analysis of all pregnancies delivered at the McGill University Health Centre during a 5-year period from January 1, 1998, to December 31, 2003 [70]. The incidence of multiple or high-order multiple pregnancies was not different among IVM (21 and 5%), IVF (20 and 3%), and ICSI (17 and 3%) pregnancies. Although cesarean delivery rates of ART pregnancies were higher than spontaneous conceptions, the incidence of cesarean delivery was similar among singleton pregnancies conceived with different treatments (IVM 39%, IVF 36%, and ICSI 36%). Likewise, the mean birth weights of all infants conceived with ART were similar among all ART groups, but lower than those of spontaneous conceptions.

In the abovementioned study, the odds ratios (ORs) for any congenital abnormality, calculated with spontaneous conceptions serving as the reference, were 1.42 (95% confidence interval (CI) 0.52–3.91) for IVM, 1.21 (95% CI 0.63–2.32) for IVF, and 1.69 (95% CI 0.88–3.26) for ICSI, respectively [70]. None of these were statistically significant. Interestingly, the odds ratio was lower for IVM than for ICSI, even though ICSI was used for all IVM cases. This suggests that the reported high congenital abnormality rate with ICSI is more likely to be due to poor sperm per se because ICSI with normal sperm used in IVM cycles did not increase the odds of congenital abnormality to the same extent.

Recently it has been reported that 1421 IVM babies born from 1187 pregnancies from 31 IVF clinics located in 22 countries, and the data were collected at the time of birth and include stillbirths but not pregnancy terminations [74]. Of the 1421 IVM infants born, there were 18 major congenital abnormalities (Table 17.1). However, it is comparable with the prevalence of major birth defects (MBDs) with the spontaneous conception per birth ranged to International Clearinghouse for Birth Defects Surveillance and Research (ICBDSR, 2011) [75]. Based on these data, it indicates that IVM procedure may not appear to pose any significantly increased risk of poor obstetric outcomes or congenital abnormalities over those already accepted with IVF or other ARTs.

17.7 Physical and Neuromotor Development

Shu-Chi et al. analyzed the chromosomal constitution and mental development of 21 children born after IVM and compared with 21 spontaneously conceived children. All of the IVM children were found to have normal karyotype and mean developmental index score, similar to controls in this small-sized study [67]. Another study of 46 IVM babies born to 40 women reported similar findings [68]. The neuropsychological development of children was assessed until 24 months and was found to be within population standards. The physical growth of IVM children seems to be similar to that of spontaneously conceived children [67, 68]. Currently available data seem reassuring and do not suggest an increased risk of congenital malformations and physical or neurological developmental delay in IVM children.

17.8 Future Goals

IVM is a relatively old topic and new technology compared to conventional IVF. IVM should be regarded as a complementary assisted reproductive technology that provides unique opportunities rather than merely a competitor of IVF. The source of immature oocytes is an important feature for the subsequent embryonic development and pregnancy, but the developmental competence of oocytes derived from the small antral follicles seems not to be adversely affected by the presence of a dominant follicle. Priming with FSH or HCG prior to immature oocyte retrieval improves oocyte maturation and pregnancy rates. The use of IVM technology can thus be broadened to treat women suffering from all causes of infertility with acceptable pregnancy and live birth rates. Patients who are at high risk of OHSS, those with unexpectedly hyper- or poor responses during controlled ovarian hyperstimulation, those with recurrent unexplained IVF failures, as well as those who are facing imminent gonadotoxic chemotherapy and are in need of fertility preservation can

Table 17.1 Obstetric outcomes and congenital abnormalities in 1421 IVM babies born from 1187 pregnancies

Characteristics from 1187 pregnancies	*Singleton pregnancies (n = 960)*	*Twin gestation pregnancies (n = 221)*	*Triplet gestation pregnancies (n = 5)*	*Quadruplet gestation pregnancies (n = 1)*
Mean gestational age at delivery (weeks + days)	37 + 4	36 + 5	35 + 2	29 + 0
No. of deliveries at >37 weeks (%)	855 (89)	60 (27)	0 (0)	0 (0)
No. of deliveries at 34–37 weeks (%)	82 (9)	132 (60)	5 (100)	0 (0)
No. of deliveries at <34 weeks (%)	23 (2)	29 (13)	0 (0)	1 (100)
Total of 1421 newborns	*Singleton newborns (n = 960)*	*Twin newborns (n = 442)*	*Triplet newborns (n = 15)*	*Quadruplet newborns (n = 4)*
Birth weight (mean ± SD) (g)	2965 ± 532	2434 ± 365	1968 ± 472	1330 ± 84
No. of LBW (%)	35 (4)	59 (13)	12 (80)	0 (0)
No. of VLBW (%)	5 (1)	12 (3)	2 (13)	4 (100)
Median Apgar score at 1 min (interquartile range)	9 (7–9)	8 (7–9)	8 (8–9)	–
No. of Apgar score at 1 min less than 7 (%)	133 (14)	31 (14)	0 (0)	–
Median Apgar score at 5 min (interquartile range)	10 (9–10)	10 (9–10)	8 (8–9)	–
No. of Apgar score at 5 min less than 7 (%)	25 (3)	5 (2)	0 (0)	–
Incidence of congenital anomalies (%)	15 (2)	3 (1)	0 (0)	0 (0)

Data reproduced from [74]
LBW Low birth weight, 1500–2500 g
VLBW Very low birth weight, <1500 g
SD Standard deviation

benefit from advantages of IVM. Although IVM as an efficient treatment resulted into several thousands of healthy babies born, IVM technology is still considered experimental by the society. Maybe, it is time to reconsider the IVM technology as an efficient clinical treatment.

Essentially, all ART laboratory procedures can be performed with in vitro matured oocytes if the need arises. The first successful IVM cycles combined with preimplantation genetic screening and percutaneous testicular sperm aspiration have already been reported [76, 77]. IVM has enabled successful treatment of patients with empty follicle syndrome in previous stimulated IVF cycles [78]. Patients can undergo several IVM cycles, and we previously reported a series of patients who achieved repeated live births with IVM treatment [79].

As the development of IVM treatment continues, an attractive possibility for increasing the successful outcome rate is combining natural cycle IVF treatment with immature oocyte retrieval followed by IVM of those immature oocytes. If the treatment processes can be simplified, especially for immature oocyte retrieval, more infertile women may be able to take advantage of these treatments. Mild stimulation IVF combined with IVM treatment may represent a viable alternative to standard treatment. As we accumulate more experience and outcome data, mild stimulation IVF combined with IVM may prove to be not just an alternative to standard treatments, but potentially first-line treatment choices [80].

Review Questions

1. LH surge induces the dominant follicle for oocyte maturation and ovulation in vivo. Oocyte maturation refers to the completion of the first meiotic division and is characterized by the extrusion of the first polar body (1PB) and formation of the secondary oocyte, both of which contain a diploid chromosome complement. Although oocyte maturation is defined as the completion of the first meiotic division from GV stage to M-II stage, the clinical definition of IVM should be considered as the procedure of immature oocytes from GV and M-I stages to M-II stage.

2. Many factors affect IVM success rates. Regardless of the different sources of immature oocytes retrieved, the most important factor affecting IVM success rate is the size of follicles.

3. Immature oocytes do not mature at the same time following IVM in culture. Practically, the maturity of IVM oocytes should be evaluated at 24 hours following culture by denuding cumulus and granulosa cells.

4. ICSI may not be required for fertilization of IVM oocytes if sperm parameters are good enough. However, practically, ICSI is recommended for insemination of IVM oocytes, because they may be inseminated two times: on egg retrieval day, 24 hours, and 48 hours following culture. Therefore, the IVM oocytes can be inseminated by ICSI with the sperm sample prepared once and used for the remaining 2 days.

5. IVM procedure seems not appear to pose any significantly increased risk of poor obstetric outcomes or congenital abnormalities over those already accepted with IVF or other ARTs.

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Design and Development of Simplified, Low-Cost Technologies for Clinical IVF: Applications in High- and Low-Resource Settings

Jonathan Van Blerkom, Christine Hennigan, and Willem Ombelet

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Learning Objectives

- How the essential principles of clinical IVF, embryo culture, and developmental competence assessment can be significantly and successfully simplified for application in high- and low-resource countries.
- How a relatively simple yet robust time-lapse microscopic imaging capacity can be added at low cost to this simplified IVF system to enable the detection of normal and abnormal embryo morphodynamics during the preimplantation stages.
- How a secure streaming video function allows contemporaneous viewing and discussion online with remote experts to aid in the technical training of embryologists for quality control and developmental competence assessment.

18.1 Introduction

With the birth of the first baby conceived by in vitro fertilization in 1978, the application of IVF technology has increased nearly exponentially for many years thereafter so as to become the worldwide infertility enterprise we witness today. The use of the word enterprise is intentional because in many respects infertility treatment has indeed become a very big business. This is not meant in a pejorative sense about an area of medicine that in reality has a very high "cure" rate for a disease, which is what infertility is considered to be by relevant medical societies and organizations worldwide, but rather one that can be costly given the clinical and technical expertise required, as well as the laboratory infrastructure needed to undertake treatment. In this context, treatment usually begins with the clinical diagnosis of infertility that is best treated by IVF followed by the following sequence of events: (i) endocrine management of the menstrual cycle to achieve some measure of controlled ovarian hyperstimulation that can be anticipated to produce multiple meiotically mature oocytes (i.e., metaphase II, MII) that are likely to be fertilization competent; (ii) fertilization in vitro and embryo culture to the cleavage or blastocyst stages for replacement in the uterus, if estimated to be appropriately synchronized at the cellular and biochemical levels to the developmental stage of the embryo at transfer; (iii) transfer by experienced clinicians, usually under ultrasound guidance; and/or (iv) preservation of embryos by cryopreservation because intrauterine conditions were found to be inconsistent with implantation or supernumerary, stage-appropriate embryos occurred which are cryogenically stored for a subsequent transfer cycle(s). In large measure, these basic elements of infertility treatment using IVF methodology have changed little over the last four decades since the first successful birth. What has changed, however, has been the progressive addition of technologies designed to improve successful outcomes, the birth of a normal baby to an infertile couple, which is the only true measure by which IVF can truly be assessed.

To this end, the progressive increase in the sophistication of the technology employed in clinical IVF laboratories derives from an underlying imperative in the field of reproductive medicine, namely, to continually strive to improve outcome and, indeed, with an increased understanding of molecular, cellular, and genetic aspects of human oocyte and preimplantation embryo biology and physiology and their requirements to function normally under in vitro conditions, this imperative has produced remarkable progress with respect to birth rates. Aside from an increased understanding of how oocytes and embryos "work," in no small measure, increased success with IVF can be directly related to improvements in laboratory design, management, culture media and equipment, and, perhaps of most significance, experienced professional clinical embryologists. But here too, improved outcomes attributable to recognizable and controllable "extrinsic" factors have come at the expense of often unavoidable increases in treatment costs to both program and patient.

In many areas of the world, continued progress in IVF of this nature is of little or no consequence to the vast majority of infertile patients in need of this treatment because even the most basic elements required to provide IVF are either unavailable or unaffordable. As described in numerous studies and reports from various international foundations, private humanitarian agencies, and governmental reports, infertility among young couples of childbearing age in so-called low-resource/developing countries is a significant and growing medical problem with important societal implications that have not been adequately addressed, if at all [1–5], such that the prospects for advanced infertility treatments remain dismal. Yet even in the "high-resource/developed countries," where IVF programs are relatively abundant and IVF births commonplace, many couples are simply excluded or "priced out" out of treatment, including those with some form of medical insurance where coverage for advanced infertility treatments is not included or severely limited for this purpose.

For those familiar with the IVF literature, it has been commonplace to see papers begin with a tribute to the original "pioneers" of clinical IVF, Robert Edwards and Patrick Steptoe. What the authors of these studies that usually come from high-volume, high-cost, high-complexity programs may not realize is that it was the vision of Robert Edwards that the methods and technologies he and his colleagues developed and so freely made available to nascent IVF programs in developed countries, be also be applied in developing countries where there was an urgent need for infertility treatment [6]. However, there are formidable infrastructural, financial, political, and personnel barriers to providing such services in low-resource settings [2]. The following section describes some of the more recent attempts designed to address the issues of affordability and accessibility that may be applicable in such circumstances.

18.2 Models for Delivering Low-Cost IVF in Low-Resource Developing countries

One approach to providing advanced assisted reproductive technologies in low-resource settings that might seem to be both practical and effective has been to promote the repurposing or donation of laboratory equipment from research or clinical laboratories, including those involved in IVF, in high-resource centers, when replacing or updating equipment. While this may be a viable option for stand-alone instruments such as microscopes, pH meters, or small clinical centrifuges, several critical challenges exist for other essential equipment in order to accomplish IVF at sites where the availability of medical grade gas for cell culture incubators is problematic or where a constant source of "clean" electricity is uncertain. Current incubators used for human IVF and embryo culture are all controlled by circuitry that can be susceptible to voltage fluctuations, which can damage or destroy microprocessors used to continuously monitor and regulate atmosphere and temperature. Frequent fluctuations in voltage (so-called dirty electricity) or interruptions in supply can be chronic in some low-resource settings requiring often costly backup power sources and line filters. This is especially relevant for CO2 and triple gas incubators where each of the abovementioned gases are individually monitored and concentrations continuously adjusted. Should these circuit boards and those controlling humidity and temperature need to be replaced and the settings recalibrated, the downtime can be quite lengthy if parts and technical service expertise are not available in a timely fashion, which is essential for IVF operations that occur under well-controlled incubation.

Another approach to delivering IVF for both high- and low-resource settings is to use the patient simply as an incubator for fertilization and embryogenesis in which gametes and embryos are physically isolated from contact with the reproductive tract in manufactured vessels placed in the uterus or vagina until removal for uterine deposition. One such device originally described by Blockeel et al. [7] was a removable intrauterine device manufactured as a hollow tube composed of a laser-microperforated silicone elastomer in which fertilization and early embryonic development occur after the introduction of gametes. Based on initial positive results from this pilot study with respect to fertilization, embryo development, and live births, a patent was issued for a similar design described as follows in the application: "The Anecova AneVivo intrauterine device which is a porous titanium chamber which allows fertilization and the first day of embryo development to occur in the patient's uterus, exposed to tubal and uterine fluids, rather than in an artificial medium, in an incubator. The device is then removed and the embryos are cultured until day 5 at which point an embryologist can select the blastocyst with the best morphology for transfer." If the device is to be used transiently as described, that is, removed on day 1 to confirm normal fertilization and embryos not reloaded and replaced in utero, development to the blastocyst stage (day 5) occurs in vitro during the subsequent 4 days. In this hybrid of "in vivo/in vitro" IVF, it would seem that the prevailing notion is the assumption that regulatory factors unique to the uterine milieu would be developmentally beneficial or significant throughout preimplantation embryogenesis if exposure occurs on the first day of embryogenesis. Because fertilization and cleavage occur in the Fallopian tube, with the former occurring on day 1 in the upper region of the tube, why uterine placement might be better suited than an artificial culture media is unclear because potential or putative beneficial uterine factors or conditions should normally be absent in this region in vivo. Nevertheless, devices such as these that use the female patient as an incubator, or for possible early exposure to yet be identified developmental regulators or promotors, may have an important role in IVF if unambiguously shown in well-designed and controlled studies to produce live births at frequencies significantly higher than currently obtained with so-called standard embryology, that is, insemination, fertilization, and preimplantation embryogenesis entirely in vitro using contemporary one-step or continuous culture media optimized for human embryo culture. At present, it is unclear whether such a device would be acceptable in a low-cost IVF setting due to cost and need for a basic laboratory if after deposition of gametes the device does not remain in utero with embryo collection on day 3, 5, or 6, but rather after the removal of fertilized eggs, culture is continued in vitro until the time selected for transfer. Alternatively, normally fertilized eggs could be returned to the microperforated tube, replaced in the uterus and subsequently removed on the day of embryo transfer.

The use of the vagina as an incubator for a manufactured vessel in which fertilization and preimplantation embryogenesis occur had been suggested and in some instances utilized during the early years of clinical IVF, but was largely abandoned because of improved commercial tissue culture incubators and culture methods, until recently that is, when a device, the INVO Cell, specifically designed for this purpose (Frydman and Ranoux [8]) was introduced commercially. In this instance, the IVF lab consists of a sterile surround, usually a small HEPA-filtered laminar flow hood, where the culture medium is prepared and pre-equilibrated with an appropriate atmosphere, sperm, and oocytes identified microscopically and for the former quantitated for insemination, and then combined in a small conical tube containing ~1 ml of medium contained within a two-piece, thick plastic chamber. The chamber is sealed with an O-ring and placed within the upper region of the vagina. Embryos are removed on a predetermined day post gamete deposition and those showing apparently normal, stage-appropriate development selected for uterine transfer by conventional means, that is, in microliter volumes bracketed by air bubbles in a catheter and displaced by a syringe.

Clinicians using this method often suggest to patients that it is more natural than IVF and acceptable to certain religions because conception and early embryogenesis occur in the patient's body, albeit within with an artificial vessel containing the same type of culture media that would be used for conventional in vitro fertilization to promote fertilization

and preimplantation embryogenesis. Neither sperm, oocytes, nor newly fertilized eggs/embryos interact with the biochemical and physical (i.e., cellular) milieu of the reproductive tract as would occur naturally in vivo, and as for being "more natural," the actual culture vessel is functionally little different from the culture tubes or culture dishes used in conventional IVF under controlled incubation at 37 °C. In addition, similar to the intrauterine device described above, the INVO Cell needs to be removed from the vagina in order to recover embryos that are then loaded into a catheter for uterine transfer. Because only one device can be placed in the vagina for incubation, the number of potentially fertilizable oocytes deposited in the actual culture compartment may be relatively small, and the corresponding protocol of ovarian stimulation should be one designed to produce a low number of fully grown antral follicles. In conventional IVF, outcome success has long been known to be strongly correlated with maternal age, the number and meiotic maturity of oocytes at follicular aspiration and because of naturally occurring increases in aneuploidy with advancing reproductive age, the extent to which recovered oocytes at metaphase II are euploid. This method may be particularly useful in programs that use natural or mild stimulation protocols to produce relatively low numbers of oocytes and potentially best suited for so-called high prognosis patients identified by age (e.g., <35), infertility diagnosis (e.g., absence of polycystic ovarian syndrome and occurrence of absent or obstructed fallopian tubes), and no medically untreatable male factor that would require ICSI (e.g., severe oligozoospermia, asthenozoospermia, or teratozoospermia). Normal development and births with the INVO Cell also call into question the physiological premise that underlies intrauterine culture in devices such as described above where early post-fertilization exposure to putative bioactive agents present in the reproductive tract are thought to be developmentally beneficial.

On the basis of recent reported outcome results [9], the INVO Cell system could be an alternative to establishing a conventional IVF laboratory that may be applicable in low-resource settings, and its use in high-resource settings in developed countries would seem to have merit if primarily a religious or psychological one for the patient. However, if incubation at 37 °C is the relevant issue for intravaginal culture, it can also be accomplished by placing the device in a low-cost, non-tissue culture incubator where temperature is fixed at 37 °C, or by other, even simpler means such as those discussed below. Potential drawbacks to using the patient as an incubator is that the normality of fertilization and early embryogenesis cannot be determined because such devices are intended to remain in place and intact until removed for embryo transfer. This so-called dark fertilization precludes the detection of certain fertilization (e.g., triploidy from dispermic penetration; maternal isodisomy associated with the occurrence of single, diploid female pronucleus owing to suppression of second polar body formation) and early embryonic abnormalities (e.g., first division from 1 to 3 cells) that would ordinarily dictate against uterine transfer and would likely go unrecognized when embryo selection for transfer occurs at later stages because embryo appearance can appear stage- and morphologically appropriate. Other morphological, morphokinetic, and temporal anomalies of developmental significance that have been identified by time-lapse microscopy and would likely go undetected after "in vivo" culture in intrauterine or intravaginal devices, and which would result in their deselection for transfer despite seemingly normal progression to the blastocyst stage, are described below.

In principle, intrauterine or intravaginal incubation relies on the notion of "self-deselection" whereby abnormally fertilized oocytes or embryos that experience most types of developmentally significant cytoplasmic defects or certain chromosomal segregation disorders fail to implant or progress to term. This is a realistic notion and the INVO Cell approach may be a viable one for clinicians and patients, if both are sufficiently informed of the potential risks of not knowing the normality of fertilization, and whether defects or abnormalities in cleavage that would likely be detected with standard IVF could compromise the likelihood of a successful outcome. At present, whether this or other methods of "in vivo incubation" associated with dark fertilization are also associated with a higher risk of adverse downstream consequences such as benign gestational trophoblast disease, the hydatidform mole (i.e., a so-called molar pregnancy) from a dispermic penetration remains to be determined although outcomes from the relatively few current reports in the literature do not indicate current concerns in this regard. However, as described below, it is our opinion that low-cost IVF can be accomplished in a physiologically defined closed system with simple means of incubation without compromising the ability to visually assess the normality of development from fertilization through the hatched blastocyst stage either by static observations or time-lapse microscopy. We suggest that such a method assures both clinician and patient that critical developmental landmarks of preimplantation embryogenesis have occurred normally and at the appropriate stage.

18.3 The Walking Egg Model

An apparent urgent need for advanced reproductive technologies to treat growing rates of infertility among young women in certain developing/low-resource countries was a central theme advanced by local healthcare providers, reproductive medicine societies, international humanitarian organizations, NGOs, and philanthropic foundations on the basis of women's reproductive rights, social justice, and equality, as noted above. Academic studies describing the abysmal and often violent treatment of infertile women by their husbands, families, and communities added a seeming urgency to bring advanced infertility methods that had become commonplace in developed countries to the most affected societies. It was against a background of formidable challenges in establishing a functional and successful low-cost IVF capacity in developing countries that a nonconventional model that

could provide such a service was developed. Parenthetically, in considering different models, applicability to couples unable to access IVF services in moderate-to-high-resource settings owing to their financial situation or to disqualifying conditions imposed on government-paid treatment was also taken into consideration so that whatever system ultimately evolved, it would have universal appeal, rather than being thought of as "low budget" for resource-poor settings only. Rather than simply duplicating the standard model of IVF laboratories common in the developing world, a significantly different approach to the laboratory aspect of delivering IVF services was devised that addressed issues of infrastructure, availability of clinical and technical expertise, and the well-recognized need to significantly improved patient accessibility and affordability for treatment.

The simplified IVF culture system (SCS) described below was originally designed by Van Blerkom et al. [10] as the laboratory aspect of the Walking Egg Program, which had been established to bring modern reproductive medicine to developing, resource-poor countries [2, 11]. The SCS was the successful culmination of several years of laboratory development and preclinical testing at the University of Colorado, Boulder, prior to results that were published in Van Blerkom et al. [10]. The work that led to the development and clinical implementation of a low-cost IVF system was done without the usual financial participation or support of government research grants, private philanthropic foundations, or public institutions that professed concern for social justice in regard to the "reproductive rights" of women. With few exceptions, such as the European Society of Human Reproduction and Embryology, in reality, the interest of most with respect to reproductive rights and equality seemed to focus primarily on population control and contraception rather than aiding infertile couples to have children. Consequently, this largely precluded from consideration projects such as the Walking Egg as appropriate venues for their support or for that matter, their attention.

18.4 The Need for a Simplified IVF Culture System

The SCS method for human IVF is designed to be a self-contained, closed culture system that is inexpensive to set up, maintain, and operate, and which largely eliminates the complex infrastructure required by the typical IVF laboratory in high-resource settings. By greatly simplifying critical aspects of the IVF culture process once gametes are in the laboratory, personnel with some clinical/medical experience should be able to successfully apply this system after relatively minimal training directed to the accurate performance of several specific steps. The inclusion of a time-lapse and streaming video capacity with coincident audio communication through the internet described below allows real-time interactions with experienced IVF laboratory personnel at remote locations so that program adopting the SCS alone or for use with the Walking Egg model [11], or one similar, can have access to the expertise of IVF specialists on an ongoing basis for purposes of training, quality control and if needed, assistance in selection of embryos for transfer. The addition of a time-lapse capacity developed for use in conjunction with the SCS is a recently added function that may be useful for selected patients, such as those with recurrent pregnancy loss or repeat IVF failure in general and where the possibility of preimplantation genetic screening is currently not an option.

Elements currently recognized as essential for successful human IVF such as culture media, pH, CO2, and O2 concentrations were addressed and solved by having a closed system that generated CO2 from a weak acid-base reaction that could be calibrated so as to produce physical and chemical conditions in a simple, sealed culture tube that are consistent with normal human preimplantation embryogenesis in vitro. Maintenance of 37 °C is a critical component of success in IVF, especially in the context of preventing adverse iatrogenic influences on meiotic and mitotic spindle integrity that could lead to aneuploidy. The use of often costly microprocessor-controlled tissue culture incubators is common practice in IVF clinics for this very reason. However, in developing and testing the SCS, it was found that 37 °C could be accurately and consistently maintained in a variety of formats, from conventional, non-tissue culture incubators to water baths, or even the unconventional, thermal beads or high efficiency thermos bottles. The SCS is intended to permit insemination, fertilization, and preimplantation development to the hatched blastocyst stage to occur undisturbed in the same culture tube until embryo removal for uterine transfer.

The design of a low-cost culture system for resource-poor settings has to consider issues associated with infrastructure that are common in developing countries, which would not be problematic for conventional systems typical of developed countries; for example, these might include (i) a reliable and constant source of "clean" electricity, that is, not subject to frequent changes in voltage; (ii) availability of replacement parts and rapid response of technical expertise to repair microprocessor-controlled instruments; (iii) on-time delivery of culture medium, medical grade gases; and (iv) sufficient clinical and technical expertise needed to maintain an IVF program after setup. The underlying challenge was to design and validate a laboratory system that while appropriate for low-resource settings could nevertheless deliver outcomes similar to those experienced in high-resource IVF settings using high-complexity laboratories.

When the possibility of establishing low-cost IVF centers in developing countries was seriously being considered in the mid-2000s by international organizations such as the World Health Organization and assisted reproductive medical societies such as ESHRE, one prevailing notion was to duplicate established IVF platforms common in the developed world with fully operational equipment donated by high-resource programs during equipment upgrades or scheduled replacements. Typically, prospective programs of this type would be hospital-based and situated in large population centers. It seemed doubtful that such a service would

truly be of sufficiently low cost to accommodate those of limited financial means or be truly assessable to patients at more remote areas. The Walking Egg Program envisaged a network of relatively smaller clinics, or satellite clinics associated with a larger central facility, that could deliver advanced assisted reproduction treatments on a comparatively local rather than singular/centralized basis as an alternative model to provide accessible and affordable IVF. However, to be cost and patient effective, an IVF laboratory needed to be developed that was relatively easy to set up and could function independently with a technical staff that while trained to perform specific tasks did not need advanced degrees in embryology or in related areas in the biological sciences (e.g., cell biology). The simplified system described below accomplishes all of the steps in the IVF process that is completely self-contained and requires no external source of medical grade gases such as CO2 or O2, and where incubation at 37 °C does not need a complex instrument designed specifically for embryo culture.

18.5 A Simplified IVF Culture System

The simplified IVF culture system is composed of two standard plain glass 10 ml stoppered medical grade borosilicate culture tubes connected by 18 gauge needles and polyurethane tubing, as shown in Fig. 18.1. One tube serves as the CO2 generator and the other for medium that is equilibrated to an atmosphere and pH consistent for human IVF. The original system described by Van Blerkom et al. [10] used standard plain glass phlebotomy tubes (e.g., Vacutainer®, BD) that were prewashed with sterile deionized water to remove a silicone coating, if present, but only in the tube containing culture medium. A second tube contained sodium bicarbonate and citric acid, which rapidly effervescences after the addition of sterile water and releases CO2 under sufficient pressure to pass it into the culture tube (Fig. 18.1a). The reader is referred to Van Blerkom et al. [10] for specific details related to reagent values and analytical methods used for the validation of culture conditions prior to clinical application. Under these conditions, the atmosphere created with respect to CO2 and O2 concentrations develops relatively rapidly, but typically the tubes remain connected for several hours at 37 °C, or overnight under refrigeration (10–15 °C). High-quality borosilicate glass tubes with tight-fitting nontoxic stoppers can be substituted for partially evacuated blood collection tubes used in the original study, but the pressure generated during CO2 formation can be sufficient to "pop" the stopper from either generating or receiving (culture) tube, so care must be taken to secure the stoppers and maintain the tubes in an upright position to avoid medium contact with the stopper, even if said to be nontoxic. Once the culture tube is secured and connection to the CO2 generator terminated, the conditions created will remain unchanged with storage at normal refrigeration temperatures, as can be visually determined by the salmon-pink color of culture medium containing phenol red as a pH reporter when returned to 37 °C. In our experience, as long as the stopper seal remains intact, the atmosphere and pH in tubes, even if stored at room temperature, remained unchanged for several years.

Experimental studies with mouse IVF and thawed pronuclear and early cleavage stage human embryos donated for research demonstrated normal preimplantation embryogenesis to the hatched blastocyst stage, with trophectoderm outgrowth after hatching on the glass surface of the culture tube observed for both species. The protocol did not permit the intentional fertilization of oocytes for the sole purpose of creating embryos for experimental rather than procreative purposes. Consequently, early pronuclear (12–14 h post insemination) and cleavage stage embryos (2–4 cells) donated to research by patients who no longer wanted to maintain them in cryostorage were used to examine the normality of development and at the blastocyst stage with respect to the following: (i) hatching frequency; (ii) ICM organization; and (iii) trophectodermal cell numbers on days 5.5 and 6.5 after conventional IVF by nuclear counts using DNA-specific fluorescent reporters.

Once the culture medium is equilibrated with the atmosphere created by introducing CO2, a stable pH between 7.29 and 7.35 exists, and if a pH indicator such as phenol red is in the culture medium, a salmon-pink tint is indicative of a proper pH for human oocyte and embryo culture. Male and female gametes can then be added to sodium bicarbonate-based continuous or "one-step" culture medium, currently available from manufacturers such as Irvine, Sage, LifeGlobal, VitroLife, Origio, and others. For the SCS, one or more COCs are added in ~15–20ul of HEPES-buffered culture medium using an 18-gauge needle (Fig. 18.1b) as originally described by Van Blerkom et al. [10] reported that with buoyant density gradient centrifugation of semen as the as the means of deriving a pelleted fraction enriched with morphologically normal-appearing, progressively motile sperm, monospermic fertilization rates similar to those reported at sperm concentrations noted above for "standard IVF" were obtained in the SCS at approximately ≤1000/ml. The long needle is gently and sterilely inserted through the stopper to a level near the bottom of the culture tube containing 1.0–1.2 ml of culture medium and the contents discharged and deposition visually confirmed under a dissecting microscope.

Typically, insemination in clinical IVF programs with "standard" embryology methodology use progressively motile sperm concentrations in the thousands/ml, often numbers ≥50,000/ml. As described by Van Blerkom et al. [10], normal fertilization rates similar to those obtained with conventional IVF were obtained with concentrations at approximately 1000/ml with sperm showing progressive forward motility characteristic of normospermic men after segregation of the pelleted fraction after buoyant density gradient centrifugation enriched in normal morphological forms. The "swim-up" method of sperm preparation first described and used for clinical IVF is also effective in enriching a population of putative fertilizable sperm if reagents for buoyant density centrifugation are unavailable

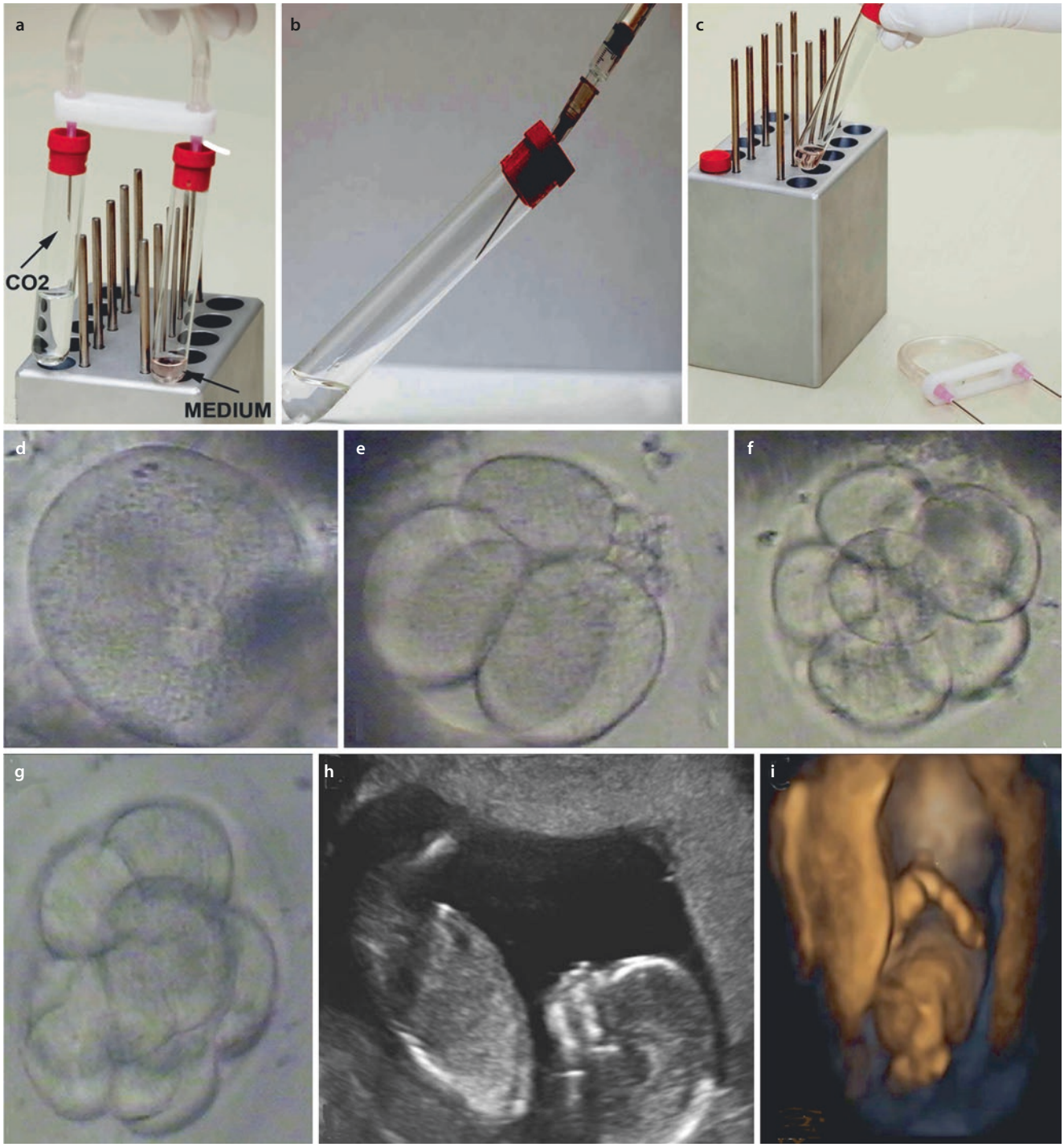


Fig. 18.1 The basis of the simplified culture system is shown in panel **a**: CO2 generated by the reaction between citric acid, sodium bicarbonate, and water passes into a second tube containing culture medium optimized for human IVF and embryo culture through the preimplantation stages. After equilibration, the pH, CO2, and O2 concentrations in the culture tube are consistent with fertilization and normal embryogenesis and remain stable virtually indefinitely as long as the stoppered seal remains intact. The addition of sperm and oocytes for IVF through a needle is shown in panel **b** and returned to an aluminum heating block **c** for culture in a water bath or simple incubator at 37 °C. Alternatively, the culture tube can be incubated in a high-efficiency thermos or in thermal beads at 37 °C. Panels **d**–**i** show the typical developmental performance of embryos conceived using this low-cost system. Panels **d**–**g** were taken in situ, that is, through the glass culture tube at the pronuclear (**d**), four (**e**)-, and eight (**f**)-cell stage. The eight-cell embryo in panel (**g**) resulted in the first birth with this system and as a fetus at 13 weeks of gestation observed by 2D and 3D ultrasound shown in panels H and I, respectively. (From Van Blerkom et al. [10])

and sperm concentrations and motility are appropriate for this technique. Of particular interest in the Van Blerkom et al. [10] report was the achievement of similar normal fertilization rates when the SCS was used for oligozoospermic individuals who would normally be classified as candidates for ICSI. In these instances, the SCS system rather than ICSI was used if a few thousand sperm showing normal motility and forward velocity could be recovered from one or more ejaculates after preparation for IVF. One potential benefit associated with the low sperm concentrations used in the SCS is that triploidy resulting from dispermic fertilization was exceedingly rare. After insemination, culture tubes can be returned to an aluminum heating block as shown in Fig. 18.1c for culture in a small, non-tissue culture incubator, a water bath, or more recently, conventional water bath where water is replaced by thermal beads (► labarmor.com) and the culture tubes completely immersed in the beads; this is a highly effective and reliable means to maintain 37 °C for the entire duration of incubation from fertilization to the hatched blastocyst stage. In either instance, multiple patients can easily be accommodated with temperature changes minimized significantly during inspections for fertilization and developmental progression by the placement of these instruments adjacent to a dissecting microscope. In addition, the placement of a common electric space heater at the air intake, or depending upon design within the interior of a laminar flow hood, can maintain the temperature at near 37 °C on the dissecting microscope surface further minimizing potential reductions in culture medium temperature during inspections (see below).

Because the SCS is a closed system, confirmation of fertilization by the presence of two pronuclei some 12–18 h after insemination can be somewhat different from standard embryology where surrounding cumulus and coronal cells are mechanically removed under direct visualization. Here, gentle vortexing for 10–30 s removes most, if not virtually all of these cells, giving a sufficient view of the cytoplasm for pronuclear detection. Where these cells are resistant to displacement, use of the long stainless steel needle described above can be introduced for mechanical denudation as would be done with conventional IVF, followed by removal in microliter volumes, of abnormally fertilized eggs (e.g., mono- and tripronuclear) and cellular debris. It is worth noting that in the experience of this author and others who have used this method, including embryologists in the Walking Egg Program, most oocytes requiring this type of aggressive denudation are meiotically immature. Other embryologists using the SCS prefer to remove all of the inseminated and cumulus-enclosed oocytes and mechanically denude using standard commercially prepared "stripper" pipets designed for this purpose in an externally heated laminar flow hood. In this instance, all normally fertilized (2 pronuclear) eggs are transferred to a new culture tube containing equilibrated medium (e.g., Fig. 18.1d), since a supply of equilibrated culture tubes is easy to have on hand. If necessary, both methods work equally well, but as noted above, ambient temperature is maintained at or as close as possible to 37 °C. Selection for transfer of preimplantation stage human embryos using the SCS is similar to protocols in standard embryology that include the following criteria: (i) stage-appropriate timing of early cell divisions; (ii) gross morphology and blastomere number and uniformity during the cleavage stages; (iii) absence of extracellular cytoplasmic fragments, and if present, their relative abundance and size, and at the expanded blastocyst stage; and (iv) cell numbers in the trophectoderm estimated empirically and when possible, by nuclear counts, and the relative size and organization of the inner cell mass [12–14].

There are several factors that recommend the SCS for use in clinical IVF for both high- and low-resource settings, where a significant proportion of couples in need of this infertility treatment are unserved because of cost or accessibility to an IVF program. The relative simplicity of design, operation, and execution with appropriately trained personnel is evident by outcome with patient costs significantly minimized with respect to laboratory charges. Where possible, treatment could include ICSI and cryopreservation by vitrification, in which the cost of the latter should also be minimized in comparison to the older-controlled rate freezing method because an expensive instrument required to perform this type of cryopreservation is no longer needed, and operator time is reduced from hours to minutes. For ICSI, use of the SCS is the same as for conventional IVF but as noted above, may be unnecessary in certain cases of oligozoospermia if a few thousand sperm with normal morphology and progressive forward motility are recovered.

18.6 Current Outcome Results with the Simplified IVF Culture System

Figure 18.1d–i shows various stages of development from fertilization (panel D) and cleavage (panels E–G) and, by ultrasonography, fetal images of the first baby conceived using the SCS [10]. It is noteworthy that the images of preimplantation development in this figure were taken in situ, that is, through the glass of the culture tube. Outcome results from the most recent IVF series using the SCS in the Genk Walking Egg Program during 2016 and 2017 are consistent with those first described by Van Blerkom et al. [10]. For example, the most current series is a prospective non-inferiority study comparing ICSI with the SCS on a selected group of patients with a least 6 oocytes recovered at follicular aspiration from women less than 43 years old, and where a total motile cohort of at least 300,000 sperm could be recovered for insemination. Sperm velocity and forward motility alone, rather than morphology, was the primary consideration for insemination in this study. Eligible patients required IVF as assessed by the standard protocol of infertility evaluation. Oocyte cumulus complexes were randomly allocated for ICSI or SCS insemination and when odd numbers, the extra complex was alternately placed in one or the other group. Of 170 cycles initiated, 163 retrievals resulted in the recovery of oocytes. Based on standard stage and morphological criteria

used for developmental competence assessments in clinical IVF [10], embryo transfers were performed on day 3 or 5. For this series, the primary outcome result was a clinical pregnancy confirmed ultrasonographically by the presence of a gestational sac and fetal heartbeat at 8 weeks of gestation. Secondary outcome measures were the fertilization rate and positive hCG prior to week 8.

Out of the 163 oocyte retrieval cycles, no embryo transfer was performed in 18 cases (11%) due to the absence of a good quality embryo (2 cases) or because of an increased risk for OHSS (16 cases), in which case all embryos were cryopreserved. No fertilization occurred in 8 (4.9%) SCS and 2 ICSI patients (1.2%), respectively. Excluding the no fertilization cases for SCS insemination that includes immature and abnormally MII oocytes, which unlike ICSI, could not be detected while cumulus enclosed and therefore eliminated for insemination, the average fertilization rate was approximately 70.8% and virtually the same for ICSI (71.2%) with only normal-appearing MII oocytes. Of the 145 embryo transfers based on "best" embryo selection criteria, 56.5% were from the SCS group and 43.5% from ICSI insemination. A single embryo transfer was performed in 73.2% of the SCS (60/82) and 69.8% of the ICSI (44/63) cycles, respectively. According to the protocol, double embryo transfers were performed for the remaining 41 patients owing to maternal age or previous IVF failure.

hCG-positive outcomes occurred in 46.3% and 39.6% of SCS and ICSI cases, respectively, which resulted in a clinical pregnancy rate of 32.9% (27/82) for SCS patients and 31.7% (20/63) for patients inseminated by ICSI, a difference that is not statistically significant. It is reassuring that the most recent outcome results are consistent with those reported from the first clinical trial [10], and moreover, demonstrate that the SCS, as used in the Walking Egg Program, is at least as effective as conventional IVF methods with respect to fertilization rates and clinical pregnancy outcomes, and in the present trial, as effective as ICSI.

18.7 Perinatal Outcomes Using the Simplified Culture System

With the above study excluded, up to June 30, 2017, 83 births have occurred since the introduction of the simplified IVF system at the ZOL Medical Center in Genk, Belgium, in 2012, which is the clinical testing site for the simplified culture system and the Walking Egg Program. In August 2017, the first baby conceived in Africa using this culture method and the walking Egg IVF model was born in Accra, Ghana: (► http://www.graphic.com.gh/news/general-news/ghana-adopts-walking-egg-technology-to-produce-baby.html). With respect to pregnancy duration, birth weight, admission to the neonatal care unit, congenital malformations, and method of delivery, data from these births were compared with the perinatal outcomes of 3265 ART babies (IVF/ICSI) born in Belgium in 2014 and reported in the Belgian Register of Assisted Procreation (BELRAP), and 3974 babies born after IVF in Flanders and reported in the Study Center for Perinatal Epidemiology of Flanders (SPE). The SCS cohort delivered 5 twin and 73 singleton pregnancies, 55 after fresh embryo transfer, and 18 after cryopreservation and transfer of up to 2 thawed embryos (Ombelet et al. 2014). The mean birth weight of fresh singletons was 3302 ± 468 grams, compared to 3211 ± 588 grams reported in BELRAP and 3328 ± 520 grams reported in the SPE registry. Prematurity (<37 weeks) occurred in 1.4% (1/55) of singletons compared with 14.2% and 9.1% in SPE and BELRAP databases, respectively. Birth weight <2.5kg was observed in 1.4% if singleton SCS babies compared to 11.6% in the SPE group and 10.4% in the BELRAP group. The Caesarean section rate was 17.5%, 23.8%, and 33.7% in the SCS, SPE, and BELRAP groups, respectively. For the 18 babies born after cryopreservation, the mean birth weight of 3827 grams was similar to the BELRAP data as was prematurity and low birth weight, occurring in only 1 of the 18 babies. Twin pregnancies delivered at 33, 34, 37, 38, and 39 weeks, with birth weights between 1250 and 3340 grams. While no congenital malformation could be detected in the SCS births and while perinatal outcomes with this system remain reassuring, according to protocols for the Walking Egg Program, strict follow-up of all babies born using the SCS method is mandatory.

18.8 Suggested Endocrine Protocols for Ovarian Stimulation Best Suited to the Simplified IVF Culture in Low-Resource Settings

A review of the literature shows that protocols using so-called mild ovarian stimulation regimens have been of value in ART settings [15]. The use of clomiphene citrate, a very inexpensive oral drug, has proven in many studies to be an effective means in IVF when a relatively small number of fertilization-competent oocytes are desired, with minimal side effects, a very low complication rate, and acceptable outcome results [15–17]. In a selected group of patients, Ferraretti et al. [18] showed that mild stimulation using a fixed protocol of clomiphene citrate (100 mg/d from cycle days 3–7) in combination with low doses of gonadotropins (150 IU of recombinant FSH) on cycle days 5, 7, and 9), and a GnRH antagonist resulted in a cumulative delivery rate of 70%. This relatively low-cost approach to ovarian stimulation is but one example that might be well-suited in a low-cost IVF setting. In other circumstances where a center can perform cryopreservation by vitrification at low cost, more aggressive ovarian stimulation to produce higher numbers of oocytes may be desirable. In this regard, it is worth emphasizing that the least costly items in the entire SCS model are the glass tubes used to generate CO2 and culture embryos. As noted above, it is a trivial matter to have on hand numerous culture tubes that are pre-equilibrated with respect to atmosphere and pH and which will retain this condition for prolonged periods. Therefore, several premade culture tubes/patient can be available to accommodate a higher number of cumulus-oocyte complexes that typically result from more aggressive (and costly) ovarian stimulation regimens, if that is determined to be best suited to a successful outcome for a

particular patient or more consistent with a program's philosophy of ovarian stimulation for ART/IVF.

In the Walking Egg model, monitoring of follicular development in an IVF cycle and the timing of hCG administration to induce ovulation can be done solely on sonographic criteria, especially with clomiphene citrate stimulation, using comparatively inexpensive, basic ultrasound equipment, thereby likely avoiding the need of expensive and frequent measurements of endocrine function determined by bioassays of hormone levels (e.g., estradiol, progesterone). Nevertheless, although very promising results from use of the SCS and Walking Egg protocols have occurred, there is still the need to perform feasibility studies that examine different ovarian stimulation options to determine which protocol(s) might be the most effective with respect to outcome, based on the patient population and their budget for treatment which, current experience suggests, will likely differ between centers adopting this low-cost IVF model.

18.9 Time-Lapse Microscopic Imaging and Streaming Video of Embryos Using the Simplified Culture System

Time-lapse microscopy is one of the more recent noninvasive methods to assess developmental competence to have entered the IVF laboratory owing to its ability to continuously record temporal, spatial, and morphokinetic characteristics of human embryos from fertilization through blastocyst hatching currently thought to indicate normal embryogenesis. Several commercially available instruments specifically designed for this purpose combine proprietary software-controlled digital image capture and embryo culture in microchambers where they remain fixed in position in a few microliters of medium (e.g., EmbryoScope, VitroLife; GERI, Genea Biomedx; ESCO Miti Time Lapse Incubator, ESCO Global). Typically, these instruments include proprietary algorithms for specific biometrics used to assess competence-related characteristics such as the timing of cleavage divisions, compaction, cavitation, and blastocyst formation as well as the uniformity of early cell divisions. Collectively, these and other characteristics have long been the mainstay of so-called static morphology-based competence assessment for selection in "standard manual embryology" [19], that is, periodic operator-dependent judgments of developmental performance during culture in IVF-appropriate incubators using largely subjective embryo "grade" or "score" assigned on the basis of time- and stage-appropriate morphology.

The extent to which an investment in time-lapse technology may improve outcome over manual embryology with experienced observers such that its use would benefit all IVF patients is currently a contentious issue, especially considering the high cost of these instruments and required culture peripherals for some [19–23]. Selection of the "best" embryo(s) for transfer at the blastocyst stage, which has become more prevalent than during the cleavage stages, is typically based on trophectoderm or inner cell mass characteristics, or both. However, the relative contribution of each to developmental competence is currently unsettled [12–14] as is the value of morphokinetic analysis in detecting aneuploid embryos with high accuracy, which would clearly be a very significant basis for routine use in IVF programs [22, 24–29]. Further confounding the routine use of time-lapse analysis is an apparent plasticity in the timing of competence-associated phenotypes identified during the preimplantation stages that while thought to be relatively fixed with respect to time after insemination/fertilization, appear to be associated with several factors including maternal age, cumulative FSH dose and method of fertilization [30]. Their findings indicate that the "origin of the embryo" can significantly confound the interpretation of morphokinetic analyses and may well need to be taken into the calculus of competence assessment for embryo selection at transfer. Nevertheless, as discussed below, the detection of certain transient, stage-related morphokinetic phenotypes that might be missed in "static" manual inspections can be highly useful in "deselecting" embryos that while seeming to progress normally when examined, have experienced earlier anomalies that reduce significantly the likelihood of a successful outcome [21, 31, 32]. It is worth noting that some of these anomalies may occur at inopportune times when embryologists are not available to perform developmentally relevant inspections.

One drawback to using current time-lapse instruments for IVF assessments is that ICSI may be required, which can add considerable cost if otherwise unnecessary because of the absence of a male factor. Unlike conventional IVF, where the tightly associated somatic cells of the corona radiata and cumulus oophorus are removed mechanically from the zona pellucida some hours after insemination when determinations of fertilization and its normality are made (i.e., for the presence of two pronuclei), imaging with commercially available time-lapse instruments requires their complete removal and ICSI if embryogenesis from the earliest stages is to be recorded. A hybrid approach uses conventional IVF in a culture dish where denudation of cumulus and corona occurs, usually some 16–21 h later, followed by transfer of normally fertilized pronuclear eggs to the micro-incubation system for individual time-lapse imaging. A potential benefit in a low-cost IVF program using the SCS is that after conventional IVF, denudation of cumulus and coronal cells occurs in situ, that is, in the same culture tube, and provides sufficient visualization of the oocyte to detect pronuclei and preimplantation embryogenesis.

18.10 Adding Time-Lapse Microscopy and Streaming Video to the Simplified Culture System Using Conventional Insemination for IVF

The current high cost of commercial instruments that integrate embryo culture with high-resolution time-lapse imaging and biometric analyses is inconsistent with a truly low-cost IVF model for both low-resource countries and for developed countries where IVF is available but, owing to cost, can prelude treatment for a large segment of the infertile population.

For the SCS in particular, integrating time-lapse would seem to be an unlikely prospect because IVF and embryogenesis occur near the curved bottom of a glass culture tube under constant 37 °C in environments where imaging would be problematic, if not impossible. Even if possible, the utility of using time-lapse in the SCS is questionable because unlike commercial instruments where each embryo is imaged separately and sequentially in specially designed microchambers, multiple embryos are usually clustered together at the bottom of a single round bottom culture tube, which could be a significant problem for image clarity and for identifying specific embryos for transfer on days 3, 5, or 6. Perhaps the most challenging issue, however, is how to reliably maintain a constant 37 °C environment during culture that will allow continuous microscopic imaging from the early pronuclear stage. Clearly, other methods used successfully with the SCS, such as water or thermal bead baths, would not allow imaging although depending on patient load, multiple simple incubators (i.e., not for tissue culture) or enclosed chambers that could house one or more microscopes may be cost-effective. While there are time-lapse instruments designed to operate within an incubator and transmit images to a nearby computer, limitations on focal length show they work best with cells/embryos on the surface of standard culture dishes such as those used in conventional IVF, and also, that one device would be needed for each dish/patient. However, our experience with such systems indicates difficulty in obtaining useable embryo resolution through the glass tube, especially at the very bottom of the culture tube where embryos reside. Finally, there is the issue of cost for specialized time-lapse software, video cameras, computers, and whether continuous streaming video to IVF program embryologists, patients, or other authorized individuals can be established; potential regulatory issues discussed below, even if addressed, might add such significant costs as to make the addition of time-lapse prohibitive in a low-cost model. Collectively, these concerns suggested that integrating necessary features for time-lapse into the tube-based simplified culture system would not be a viable option, or able to offer the same image resolution engineered into commercial instruments needed to characterize morphokinetic and morphological features that maybe related to developmental competence as preimplantation embryogenesis progressed from the pronuclear stage.

The following discusses how these issues were addressed and led to several iterations that could be integrated into the low-cost SCS/IVF model to produce time-lapse data highly suitable for competence assessment.

18.11 Temperature Regulation in an Open Incubation System for Time-Lapse Microscopy

The foremost challenge from both a safety and potential regulatory perspective encountered during the development of the SCS was to demonstrate that a constant ambient temperature of 37.0–37.1 C could be accomplished outside an incubator engineered for tissue culture or a simple one that only maintained a specific temperature. The same issue confronted the addition of a low-cost time-lapse capacity as continuous imaging and recording from insemination to the hatched blastocyst stage (i.e., for some 6–6.5 days of culture) through the glass SCS culture tube, which required its placement directly above an objective lens on the stage of an inverted microscope (Fig. 18.2b). Several iterations of external incubation from the least expensive, a simple fan-driven electric space heater for each microscope to a similarly heated Plexiglas enclosure that could accommodate several microscopes, to setting up a dedicated 37 °C mini-warm room in which embryos from multiple patients could be monitored were tested. As described below, the simplest iteration as shown in Fig. 18.2 uses a common electric space heater delivering up to 4000 btu/h with high, medium, and low presets to a single microscope. Although the easiest to setup and maintain, it may, however, be the most contentious design with respect to assuring regulatory agencies of its safety and effectiveness.

When the heater is properly positioned at a predetermined height and distance from an inverted microscope (e.g., Fig. 18.2a, b), a curtain of heated air surrounds the microscope and continuously maintains an ambient temperature of 37 C (± 0.1–0.2 C) at the microscope stage where embryo culture in the SCS tube occurs directly above the objective lens (Fig. 18.2b, asterisk, Fig. 18.2c). A small dark room where the system can remain largely undisturbed during culture should be dedicated for this purpose. A logging thermometer with accompanying software that transmits temperature at predetermined intervals for graphing provides a continuous level of assurance that temperature remains constant during the entire imaging/recording process (e.g., Omega HH802W, Omega Inc., Norwalk, CT). Further assurance of thermal stability can be achieved by the addition of a bench-top temperature controller that immediately shuts the heater off should temperatures between 0.1 and 0.2 °C above a 37 °C set-point occur unexpectedly, and then turns on once the normal temperature is detected (e.g., BriskHeat, SDC120JCA, BriskHeat Inc., Columbus, OH). The thermocouple connected to the temperature controller is placed at the site of embryo imaging or directly on the culture tube as described above. Temperature controllers with similar sensitivities that monitor and regulate multiple zones are readily available to control temperature on multiple microscopes. Indeed, temperature monitoring with a digital thermometer, preferably with logging functions, can be done by positioning the tip of a thermocouple at the site of embryo culture for added assurance of thermal stability (asterisk, Fig. 18.2c) or in a sham culture, where the thermocouple is in the culture medium in a stoppered SCS tube, or both. Our studies have shown that when largely left undisturbed, temperature remained stable within the 37 °C range for at least 14 days. The right-hand arrow in Fig. 18.2b shows the temperature measured with an alcohol thermometer that is slightly below 37 °C, while the digital thermometer in Fig. 18.2b reads 37.2 °C. Experience with this time-lapse configuration has shown that for this purpose, digital measurements are more accurate than those from standard analog thermometers. Indeed, with a long thermocouple whose tip is placed where

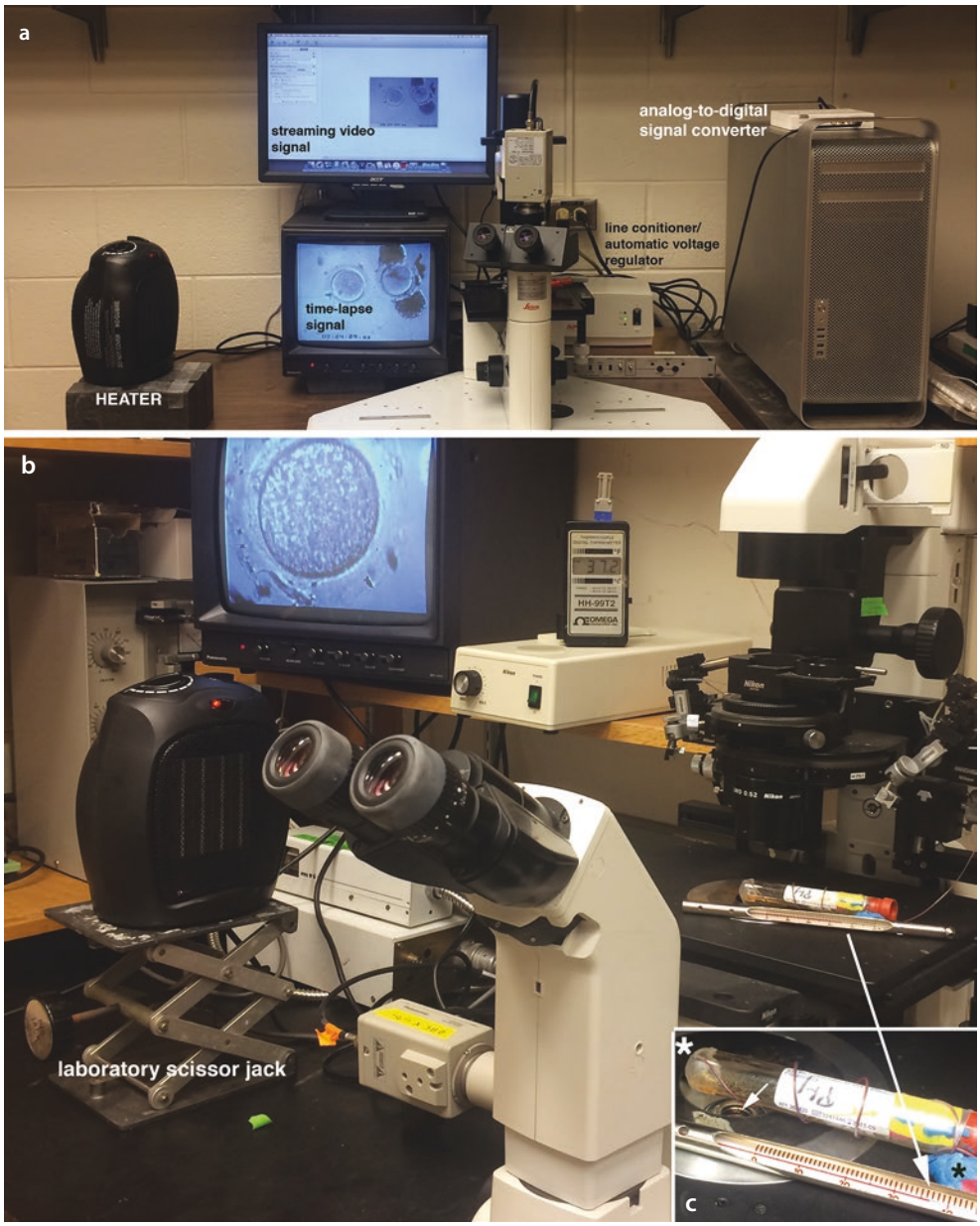


Fig. 18.2 The setup for time-lapse microscopy and streaming video in its simplest iteration for use with the simplified culture system is shown in panel **a**. Each of the components is labeled and a constant 37 °C temperature at the site of culture on the microscope stage (indicated by arrow in insert extending from the microscope stage) is maintained by a curtain of heated air from a common electric space heater placed at a predetermined distance and height from the site of culture above the objective lens. The lower monitor shows two pronuclear eggs in the process of being imaged by time-lapse, while the upper shows the same image being streamed through the internet to remote observers on a secure website. Figure **b** shows how the thermocouple wire from either a temperature controller or digital thermometer (shown in **c**) is wound around the culture tube with the thermal sensing tip located at the site of embryo culture. A standard alcohol-based analogue thermometer is shown at the same location with a temperature measurement of 36.5 °C, compared to the 37.2 °C with the digital thermometer in **c**. These values are within the 37 °C acceptable range for each type. In this instance, both embryos developed to the hatched blastocyst stage after 6 days of culture

embryos are being recorded, the actual thermometer does not have to be in close proximity to the microscope, or for that matter, even in the same room.

Because embryo culture in this iteration of a time-lapse capacity has the culture tube sitting on a microscope stage above the objective lens, it is essential that a backup power source be available to immediately supply electricity as temperature will drop in this type of open incubation and the potential benefits of undisturbed embryo development in the closed SCS would likely be lost if prolonged. Common APC units are sufficient to maintain the peripherals in this system such as computers, video cameras, and microscope light source. However, as with any IVF unit, backup electricity for high-demand instruments such as laminar flow hoods and incubators is a necessity, and the same would be required to keep a constant 37 °C whether using the air curtain described above for a single microscope or a Plexiglas chamber containing several microscopes communicating with a computer and

ambient temperature controlled as described above. Likewise, if voltage fluctuations are a chronic issue, an inexpensive in-line filter that regulates voltage will maintain normal computer, temperature regulation, and video camera functions during time-lapse imaging, as shown for this simplest iteration in Fig. 18.2a.

18.12 Time-Lapse Microscopy Beginning at the Pronuclear Stage After Conventional Insemination or ICSI

Time-lapse imaging for the SCS was designed to record and stream online preimplantation embryogenesis from conventional insemination or ICSI in cumulus and corona cell-free embryos from pronuclear through the hatched blastocyst stage in the same culture tube. As described previously [10], denudation of cumulus and coronal cells by gentle vortexing to check for fertilization is typically effective to visualize pronuclei. In instances where cumulus and coronal cell removal is problematic or incomplete, insertion of the same 23-gauge needle used to deposit gametes (see above) can be used to mechanically denude oocytes by standard "stripping" procedures. This is usually effective for creating a cell free or clear area for time-lapse imaging as cellular remnants can be removed with the same needle, as can abnormally fertilized eggs, such as those containing a single or three or more pronuclei. Despite an extra step in the SCS protocol with conventional IVF, removal of all normal pronuclear eggs in a small volume of medium followed by transfer to a fresh tube of equilibrated medium and repositioning of embryos as described above is the preferred method for prolonged time-lapse recording of multiple embryos. The simultaneous time-lapse recoding signal and streaming video function of this time-lapse system adapted for the SCS is shown in Fig. 18.2a for two pronuclear stage human embryos, one of which has been completely denuded of cumulus and coronal cells and the other with two regions of the intact cells whose presence does not interfere with pronuclear detection. The streaming function for remote viewing in the IVF unit or by another authorized observers online is secured though an encrypted website to which only authorized users would have access.

Typically, as many as 6 pronuclear stage embryos can be effectively imaged by time-lapse at any one time if embryo transfer is planned for day 2 or 3, that is, cleavage stages. After gentle embryo deposition at the bottom of the culture tube as described above, it is kept in a vertical position for 5–10 min at 37 °C followed by a slow tilting to a near horizontal position directly above the objective lens on the stage of an inverted microscope warmed to 37 °C by a curtain of heated air (see Fig. 18.2b). Once embryos are located, the culture tube is secured in place at an angle of ~10° to the horizontal with a small piece of modeling clay placed near the stopper (asterisk, Fig. 18.2c). If necessary, closer embryo clustering can be achieved by moving the stage in a gentle rocking motion using the X- and Y-axis stage adjuster. Alternatively, centrifugation at low speed (≤100 rpm) in a common clinical fixed angle centrifuge for ~20 s usually places embryos in juxtaposition near the bottom of the tube, and they tend to move together to its side when placed nearly horizontal, which promotes imaging with minimal distortion from the glass. After final location for imaging, embryos typically remained fixed in position during recording. When embryo culture is extended to day 5 or 6, a maximum of three embryos/tube is preferable and some repositioning may be required during blastocyst expansion and hatching owing to embryo-generated movement during emergence from the zona pellucida and continued blastocoel expansion when free. Up to day 4, when compaction occurs, individual embryos can usually be identified for selective purposes based on location and embryo-specific morphokinetic and morphological characteristics. During blastocyst formation, this generally becomes more difficult if more than 3 expanding embryos are present owing to their natural morphokinetic activity that generates movements, which can place one or more embryos or the ICM out of the field of view.

Our studies of different optical platforms (brightfield, phase contrast, differential interference contrast) showed that imaging of embryos taken through the glass culture tube was surprisingly clear and largely distortion-free under brightfield illumination with low power 5X and 10X plan-apochromatic objectives with high numerical apertures. Imaging can be performed on basic inverted microscopes (e.g., Leica, Nikon, Olympus, etc.) with only brightfield illumination and appropriate low-cost lenses. Long working distance objective lenses are preferable for pronuclear through hatched blastocyst stage because they provide the depth of field necessary to resolve fine cellular and nuclear details, as discussed below. For use in low-resource settings, used or donated inverted microscopes may be available from a variety of sources, including IVF programs that update or change equipment, especially those that utilize commercial time-lapse instruments.

18.13 Time-Lapse Microscopy and Streaming Video Under Very Low Light Illumination

With respect to cost versus quality of imaging of human embryos in the SCS model with brightfield optics at very levels of low illumination, relatively inexpensive analog or digital monochromatic cameras designed for surveillance purposes are readily available and can provide sufficient high resolution needed to detect morphokinetic activity during from the pronuclear to the hatched blastocyst stage. Because embryos are colorless, we show examples from an analog Panasonic WV-BP110 CCTV monochromatic camera (i.e., black and white; Fig. 18.2a). Transformation of an analog to a digital signal for computer-based video streaming and time-lapse imaging requires an analog-to-digital converter (Fig. 18.2a, e.g., Canopus ACDV110: Grass Valley, Hillsboro, OR.), which is unnecessary with digital cameras but while usually more costly, did not provide enhanced

resolution or greater depth of field in our experience. Placement of a 630 nm long pass filter in the optical path eliminates light in the far blue and ultraviolet range emanating from a halogen light source and as an added benefit, noticeably increases the contrast of the black and white images. Filters of this type are readily available online in diameters and thickness that can be placed in the light path of inverted microscopes. In practice, low light microscopy is achieved by closing the field and condenser diagrams until an embryo image on the video display is just detectable without loss of resolution due to increased graininess; this was found to be sufficient to provide developmentally relevant morphokinetic details of embryo performance from pronuclear to blastocyst stages that are similar to what is obtained from commercial instruments and used for purposes of embryo competence assessment. Under these conditions of illumination, which measured <0.55 lux at the site of embryo culture, red light is barely visible to the naked eye. As discussed below, time and stage of development from pronuclear to fully hatched blastocyst stage in the SCS, with or without time-lapse using sibling embryos, were similar, if not indistinguishable, indicating that while this iteration of the SCS time-lapse platform has logistical limitations, it can be an effective one for embryo competence assessment in a low-resource setting. As noted below, illumination does not have to be continuous but can be programmed to be on or off at predetermined intervals.

18.14 Time-Lapse Microscopy and Video Streaming Software for Use in the Simplified Culture System at Low Cost

As with most time-lapse imaging software available online, including those available at little or no cost, user information and manuals for setting image capture parameters (time, brightness, contrast, etc.) and establishing a unique website to stream images to external observers (e.g., patients, embryologists) are usually sufficiently thorough and well-written for an individual versed in basic information technology to incorporate this capacity into the SCS and set up a secure streaming video website. This is the case for each of the software programs noted below where live images during 6 days of preimplantation embryogenesis were securely streamed to interested observers on several continents in order to demonstrate the expected resolution and clarity of imaging, and the type of morphokinetic information that could be obtained to characterize embryo performance for selection purposes. One very useful feature usually included in the software is the ability to stop recording if no light signal from the microscope is detected and when detected, resume imaging. This allows recordings to be discontinuous and timed to when certain developmentally significant benchmark events during preimplantation human embryogenesis would be expected to occur, such as the first cell division, morula to cavitation, blastocyst formation, and hatching. In this way, light exposure during prolonged and continuous time-lapse recording, as would occur from pronuclear to blastocyst hatching, can be further minimized by having the microscope light source interface with a simple event timer with multiple preset-time points. Alternatively, some software allows preprograming of recording set to times that bracket expected developmentally relevant events currently thought to be predictive of normal blastocyst formation and implantation, such as the initial cleavage divisions [20].

The availability of software from multiple online sources as either freeware or at very low cost was a surprising outcome in our investigation of programs that could be used for time-lapse application in the SCS. While most were found to be designed for video surveillance and consequently had limited functionality in cell biology, including documentation of human embryo morphokinetics, as of this writing, the following were found to be suitable for use with the SCS in the iteration described here: EvoCam5, Security Spy, SwiftCapture, Sky Studio Pro, Chronolapse, VLC, SpyCam, and Gawker. However, with respect time-lapse/streaming capabilities, the EvoCam5.0 (▶ evological.com), and SwiftCapture, SecuritySpy (▶ bensoftware.com) provided the most useful features, functions and high-quality image capture under very low light conditions. Each of these programs can accommodate and simultaneously display inputs from several microscopes, which may benefit IVF programs that want to monitor and record the embryos of several patients simultaneously while retaining a low-cost structure. Other important considerations in the selection of software include support for a wide variety of digital and analog video cameras, such as the basic Panasonic monochromatic video camera noted above (◘ Fig. 18.2a), auto brightness and contrast functions, and the capacity for discontinuous recording. This last feature can be very useful if the software can be programmed to turn on or off recording at predetermined times and intervals. As noted above, this can be done in concert with the microscope's light source controlled by a simple multi-event timer, allowing time-lapse recordings to occur around the time when morphokinetic events considered relevant for competence assessment should occur. These events would normally include pronuclear formation and dissolution, first and second cleavage divisions, morulation, cavitation, delineation of the inner cell mass, and blastocyst expansion and hatching.

Time-lapse data is saved as JPEGs or as Quick-time files, which facilitates image processing, for playback, if necessary. For practical purposes, images are usually captured at 5-min intervals and in our studies during development of this application, for up to 165 h (day 0.5–7). All images from time-lapse sequences discussed and shown below are "raw," that is, as recorded and without any post-capture digital imaging processing.

Video monitoring provides an important clinical dimension to the SCS because it allows embryologists and clinicians within the IVF unit to follow the progression of preimplantation development in real time without disturbing embryo culture until transfer with embryo selection for transfer based on morphokinetic information obtained during culture. The

video streaming function also allows outside observers to securely view the same embryo for the purposes of quality control (see Fig. 18.2a), training or when combined with simultaneous communication (e.g., Facetime, Skype), and to assist technical staff in embryo grading for transfer or cryopreservation. In this way, low-cost programs can provide a robust online platform for low-resource/developing countries that can include remote and secure internet access for others.

It is important to emphasize that unlike the commercial time-lapse instruments currently in use in clinical IVF, this system has a limited capacity with respect to the number of patients and embryos that can be monitored at the same time. This needs to be understood in the context of the regime of ovarian stimulation, that aggressive gonadotropin stimulation protocols designed to produce large numbers of antral follicles and consequently high numbers of embryos is largely inconsistent with both the low-cost SCS model and this time-lapse application.

18.15 How the Application of Time-Lapse Microscopy Contributes to Embryo Selection to Improve Outcome Using the Simplified Culture System Platform

With relatively simple optics and a basic inverted microscope in a well-controlled thermal environment, fine cellular details during undisturbed culture from the pronuclear through hatched blastocyst stage can be resolved to offer morphokinetic and temporal information for embryo selection. Although not shown, common fertilization abnormalities at the earliest stages of human embryogenesis such as triploidy (three pronuclei) or activation without second polar body formation (single polar body and single pronucleus) are as easily detectable as is the case with properly timed static inspections for fertilization by conventional IVF culture. Panels 1–6 in Fig. 18.3a are individual images taken from a time-lapse sequence that began with pronuclear detection 14 h post insemination by conventional IVF and after cumulus and corona cell denudation (PN) and continues through pronuclear membrane dissolution that precedes the first cell division (arrows, panels A5 and 6). These images illustrate the level of resolution attainable in this system with imaging through the glass culture tube that is sufficient to resolve fine cytoplasmic and nuclear details such as the size, location, and number of nucleolar precursor bodies (*n*) within the male and female pronuclei (Fig. 18.3a1–4). Of particular practical interest in this sequence is a somewhat earlier breakdown of the juxtaposed pronuclear membranes in the embryo on the right-hand side in panels 3–5. Asynchrony in pronuclear membrane breakdown, (often termed syngamy), may reflect differences in the time of sperm penetration or intrinsic differences in oocyte activation that may have downstream developmental relevance if the delay is significantly prolonged rather than by just an hour or two. However, nucleolar characteristics (often referred to as nucleolar precursor bodies) have long been suggested to be relevant early biomarkers of developmental competence when observed in static inspections at the first check for pronuclei after IVF [33], but changes in their location and orientation during the pronuclear stage may be of equal relevance and, in practice, detectable only by time-lapse imaging [34, 35]. In the same respect, the orientation of the pre-breakdown pronuclei with respect to the first or second polar bodies, or both, has also long been suggested to be an important biomarker of developmental competence (see Papale et al. [33]) that may be best detectable by time-lapse imaging owing to their own morphodynamic activity in the newly fertilized human embryo.

Abnormalities at the first cleavage division(s) that may indicate developmental abnormality, incompetence, or the likelihood of mitotic aneuploidy that are routinely detectable in commercial IVF time-lapse systems are also observable in this iteration of a low-cost time-lapse application for the SCS and include the following: (i) nonuniform, highly asymmetric, or abnormally delayed early cleavage divisions; (ii) a first cleavage division from one to three cells; (iii) cytokinesis of one or more blastomeres that is significantly premature with respect to the normal human blastomere cell cycle; (iv) multi-nucleation; (v) degree of fragmentation and perhaps the strangest anomaly; and (vi) stage reversion/ reverse cleavages. Panels 1–6 in Fig. 18.3b show apparently normal pronuclear formation and dissolution between 14 and 21 h following conventional IVF (accessory sperm, SP evident in zona pellucida). However, some 100 min later, the first cleavage division clearly produces three blastomeres (panel B7), a phenomenon normally associated with dispermic fertilization (triploidy) and a tripolar spindle. Cleavage divisions during the subsequent 16 h resulted in an apparently normal six-cell embryo with uniform blastomeres and no evidence of fragmentation (panel 8). Static inspections, first to determine pronuclear status and later whether cleavage on day 2 or 3 was normal, might have missed this abnormality in early cytokinesis that would have likely produced a normal-appearing embryo classified as transferable but unknowingly, with a high probability of being aneuploid.

Panels 1–10 in Fig. 18.4a show another advantage of time-lapse analysis during early cleavage detectable with the described application for the SCS. In this instance, an abnormality in cell division timing affecting one blastomere occurs at the two-cell stage, with potential downstream consequences of mitotic chromosomal aneuploidy/mosaicism. This sequence begins at pronuclear membrane dissolution at 19 h 20 min after conventional IVF (arrow, panel A1). The first cleavage division at 22 h seems normal and results in what appears to be a typical two-cell embryo with even blastomeres and no evident extracellular fragments (panel A3). However, about 2 h later, the nascent blastomere indicated by an asterisk in panels A2 and A3 divides again forming three cells, each of which appears mononucleated (*N*, panel A4, arrows panel A5). The first cleavage division for the other blastomere occurs at 36 h (panel A6) and produces a transient five-cell embryo (one cell out of plane of focus in panel A7) and at least three are mononucleated (arrows; panel A7). Although subsequent cell divisions seem to be largely normal

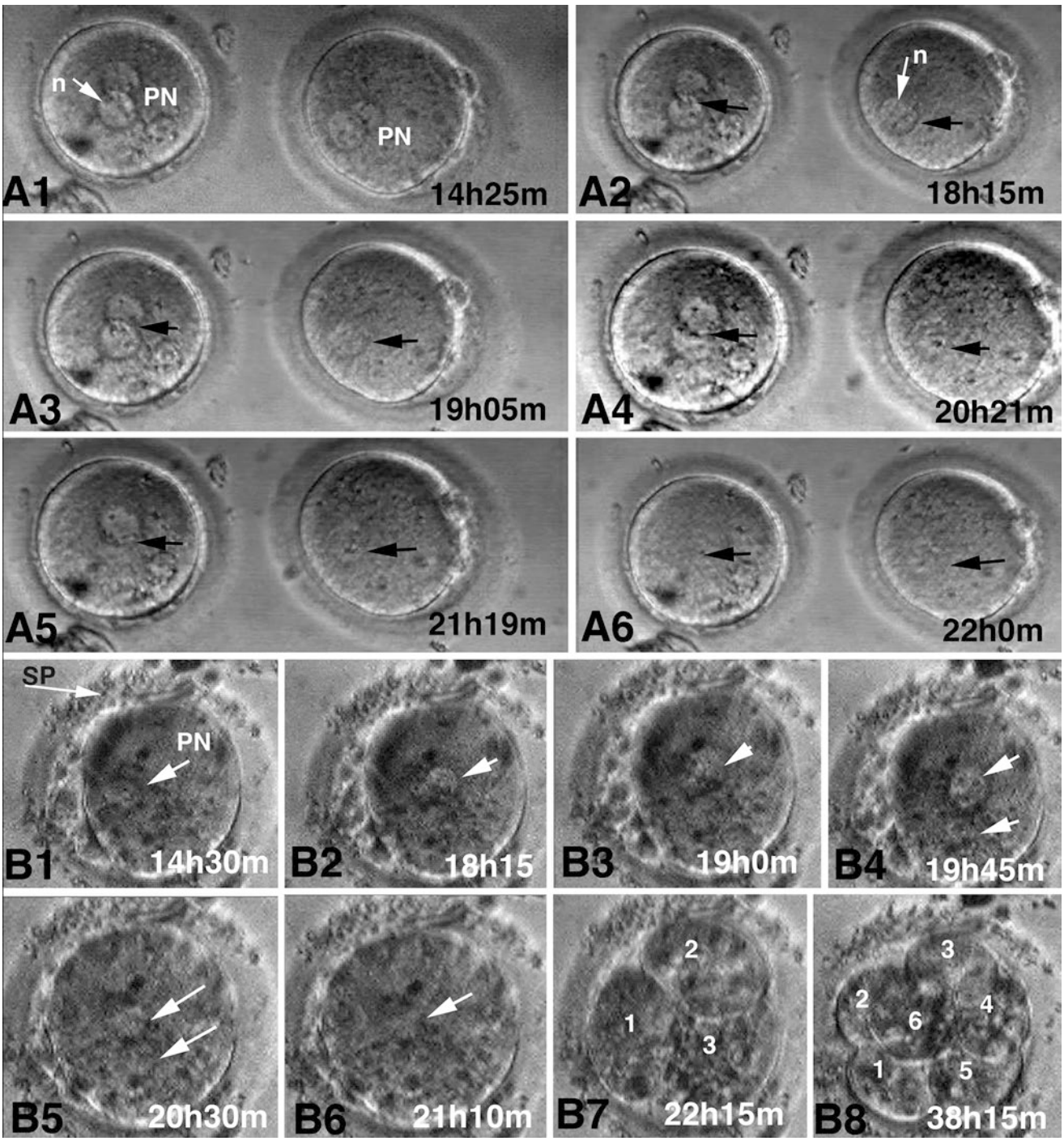


Fig. 18.3 Selected sequence from a time-lapse video showing normal pronuclear juxtaposition (panel **a**1) after conventional insemination in the glass SCS culture tube, followed by pronuclear membrane (PN) dissolution for the embryo on the right (panel **a**2) and somewhat later for its sibling embryo on the left (panel **a**5). These images were recorded near the bottom of the culture tube positioned directly above a 10X objective lens using brightfield optics and filtered 630 nm, low light illumination (0.05lux). The resolution was sufficient to detect individual nucleoli (nucleolar precursor bodies, *n*) in each pronucleus. In Fig. **b**, the oocyte was naturally darker, probably due to high lipid content. Pronuclear evolution (PN, arrows, panels **b**1–4), membrane dissolution (panels **b**5 and 6), and the first (panel **b**7) and second cleavage divisions (panel **b**8) are shown in this sequence. Of particular note is the first division into three cells (panel **b**7) followed in 16 h by a second division that produced a normal-appearing six-cell embryo (panel **b**8). Time from conventional insemination (SP, sperm) is shown at the lower right

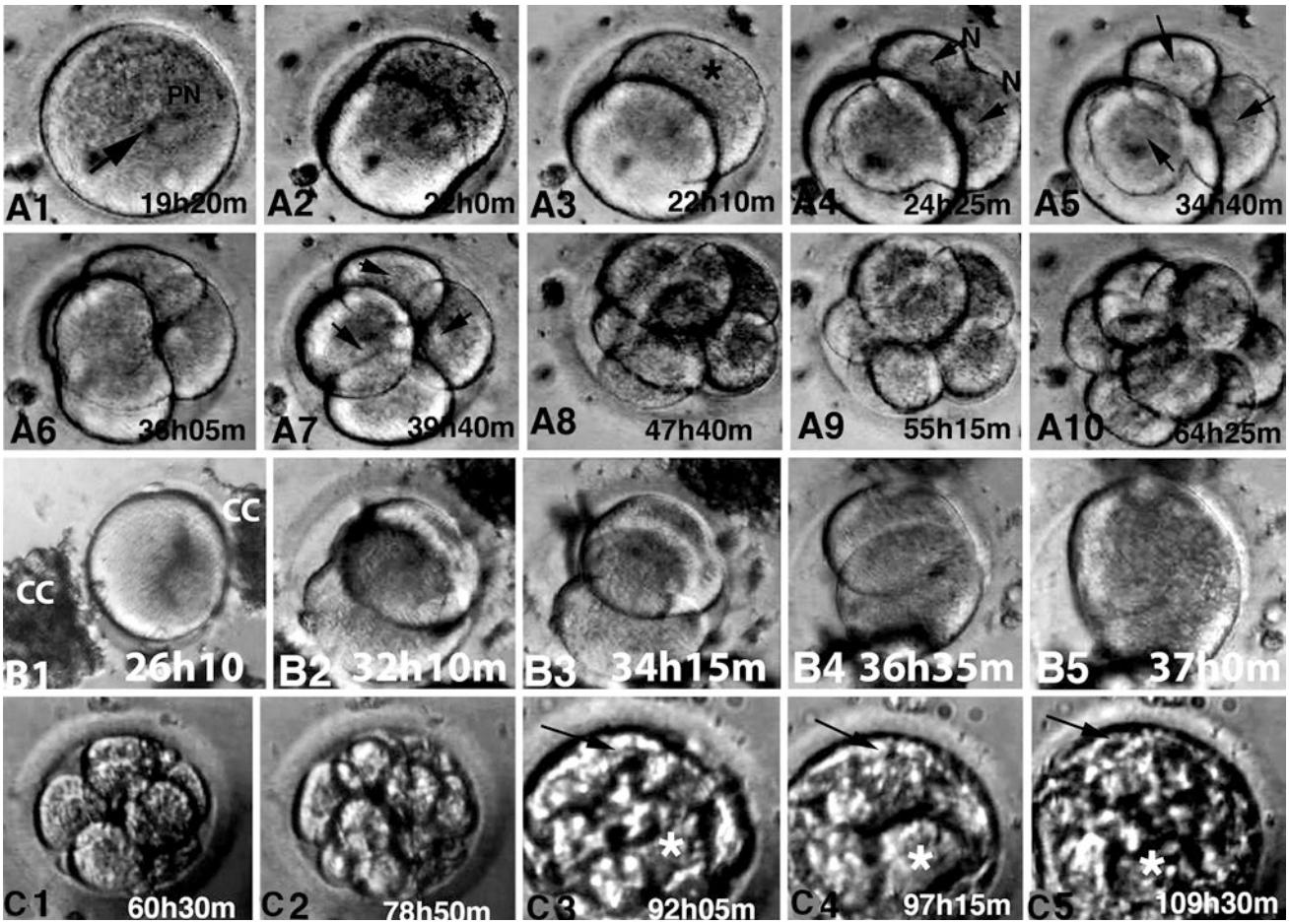


Fig. 18.4 In panels **a**1–10, a time-lapse sequence beginning at 19 h after insemination reveals an abnormal pattern of early cleavage division. Panel **a**1 is a normally fertilized egg near the end of the pronuclear membrane dissolution stage, which is followed by an apparently normal first cleavage division some 3 h later (panels **a**2 and 3). However, what time-lapse revealed was that 2 h later, the nascent blastomere indicated by an asterisk in panel **a**3 divided again into three apparent cells (panel **a**4), each of which was clearly mononucleated (panel **a**5). At 36 h, the second blastomere divided normally producing a five-cell embryo (panel **a**6). The three cells from the prematurely dividing blastomere shown in panel **a**5 were still mononucleated (indicated by arrows in panel **a**7). The fifth cell, the product of an apparently normal cell division of the second blastomere in **a**5 is out of the plane of focus in panels **a**6 and 7. Continued cell divisions produced blastomeres of largely even size (panels **a**8 and 9), but at 64 h, the embryo appears morphologically normal and by static evaluation, would likely have been considered stage appropriate for transfer (panel **a**10). The panels in Fig. **b** show the ability of this time-lapse microscopy system as applied to a simplified culture system to record so-called reverse cleavage. The post-pronuclear dissolution stage embryo in panel **b**1 undergoes an apparently normal first cell division as shown in panels 2–4. However, approximately 1 h after the completion of cytokinesis (panel 5), an abrupt reversion to a single cell is observed representing a phenomenon termed "reverse" cleavage (cc, cumulus cells). The panels in Fig. **c** show normal cleavage divisions (panels 1 and 2), morulation (panel 3), and early cavitation indicated by internal fluid accumulation (asterisks, panels 3–5). The arrows in panels 3–5 indicate a progressive thinning of outer cells that will later become trophectoderm. Times from conventional insemination shown at the lower right

(panels 8 and 9), by day 2.5 (panel A10), the eight-cell embryo appears stage- and time-appropriate and would likely be classified by a static inspection as morphologically normal for a day 3 transfer.

So-called reverse cleavage, the reversion of presumably separated daughter blastomeres to the previous stage. Was clearly detectable with this imaging system, as shown in Fig. 18.4b, panels 1–5. Panel B1 is a post-pronuclear breakdown stage embryo that is divided into an apparently normal-appearing two-cell embryo (panels 2–4). The partial rotation of this particular embryo evident in these selected images was due to two clusters of motile cumulus cells on opposite sides at the one-cell stage (cc, panel 1) that had not been removed completely during the denudation step. However, the two-cell embryo (panel 4) suddenly reverts to a one-cell stage (panel B5) in about 25 min. In some infrequent instances, continued culture in situ showed one or more cell divisions some hours later that resulted in what could be classified as a morphologically normal four-cell embryo. However, preliminary cytogenetic findings from the few embryos examined to date showed all were chromosomally abnormal (mosaic).

Figure 18.4c shows the normal process of compaction (panels 1–3) and cavitation (panels 3–5) at the morula stage where fluid accumulation (region indicated by an asterisk) begins the formation of what will later be an expanded blastocyst cavity or blastocoel. The resolving power of this time-lapse imaging system is clearly illustrated during the process of blastocyst formation and hatching. The panels in Fig. 18.5 A and B are representative images of a time-lapse sequence taken in a fully expanded blastocyst during hatching with both trophectoderm and inner cell mass clearly delineated in figure b. Of particular note with respect to the quality of imaging obtained through a plane glass culture tube is the ability to detect cell division in the trophectoderm, as shown in panels A1 and A2. The dark region indicated by an arrow in panel 1 is a trophectoderm cell nucleus in cytokinesis and in panel 2, the two nascent daughter nuclei after the completion of cell division (arrow).

Panels 5B1–8 are images from a time-lapse sequence of blastocyst hatching on day 5.5 of culture after conventional IVF in the SCS system. In addition to potential morphokinetic information, this sequence demonstrates the clarity of imaging that can be obtained in this simplified time-lapse system without additional image processing or enhancement, as evidenced by the cellular detail observed as the embryo progressively emerges from within the zona pellucida (white arrow). It also demonstrates significant differences in the timing of complete emergence for two sibling embryos that appeared stage- and time-appropriate during earlier preimplantation stages. Although the embryo on the left-hand side is somewhat smaller than its sibling due to a cycle of collapse during emergence, after hatching is complete, continued expansion of the blastocoel results in embryos of a similar diameter and appearance.

The selected images in Fig. 18.5c are from a time-lapse sequence of preimplantation embryogenesis beginning at the eight-cell stage (panel 1) and continuing through subsequent cleavage divisions (panel 2), compaction/morulation (panels 3 and 4), early fluid accumulation during cavitation (panels 4 and 5), blastocoel expansion (panels 6–9), and finally, failed hatching (panels 9–14). Despite evident zona thinning (small asterisk, panels 2, 5–6, and 10) during blastocoel expansion denoted by a large asterisk in panels 5, 6, and 8, with delineation of the ICM, several consecutive cycles of blastocoel collapse and re-expansion occurred with a final collapse at 149 h post insemination (panel 10; day 6.2). Depending upon the position of the embryo at the start of culture in the SCS, slender cellular processes emanating from one or more clusters of trophectodermal cells often penetrate and extend through the zona pellucida (black arrow, panels 7–14). These cellular extensions can be highly motile and are likely the kinetic structures termed "zona breakers" by Sathananthan et al. [36] owing to their suggested function in creating the initial rent in the zona that is permissive for hatching. Despite their presence, hatching in the embryo shown in Fig. 18.5c failed, despite the persistence of kinetic activity for these transzonal structures for up to 24 h.

For purposes of selection for transfer on days 5–6, usually three expanded blastocysts can be observed at one time with a low power objective lens (e.g., 5X long working distance) and can usually be identified individually after removal from the culture tube by virtue of (i) whether they are intact, hatching, or hatched and (ii) appearance and organization of a detectable ICM (e.g., compact, nodular, laminar, or disorganized). Gentle side-to-side movement of the microscope stage is sufficient to bring the ICM of a hatched embryo into focus for morphological assessment. In this context, according to certain ICM grading schemes the embryo on the right-hand side in panel 8 would be classified as high grade and the one on the left, at a lower grade with respect to the selection for single embryo transfer [12–14].

Whether terminally collapsed embryos such as shown in panel C14 could re-expand and emerge from the zona pellucida after laser-assisted hatching either in situ or by removal and placement in conventional culture was examined in preliminary studies. None of the 24 embryos showing the panels C11–14 phenotype (i.e., with several previous cycles of collapse and re-expansion) benefited from this treatment by re-expanding. This finding tentatively suggests that such embryos maybe terminally compromised with respect to development competence and would be unlikely to either implant or progress through gestation after transfer. However, while the validity of this interpretation requires further study, it does support the use of time-lapse with the SCS for multiple patients using a multiple microscope iteration as described above.

18.16 Limitations and Future Prospects

The intended "take-home message" from this chapter is that it is possible to simplify what would normally appear to be complex aspects of IVF into a platform suitable for an IVF setting in most locations in developed and developing countries. While primarily intended for patients that do not unambiguously require ICSI, this culture system, with or without time-lapse/streaming video, can be used to reduce laboratory costs to a significant extent and thereby allow more patients to afford or have access to treatment. The time-lapse system described here and methods for maintaining a constant and normal culture temperature, while unconventional, represent only one possible template for introducing a "high-tech" method in a low-cost setting. As noted above, one possible iteration that may be a reasonable and cost-effective means for its adoption in a low-resource setting that would likely address or comply with potential regulatory concerns is to have several microscopes in a common temperature-controlled enclosure, in essence a mini "warm room," with image transmission for recording and live streaming using available software such as described here.

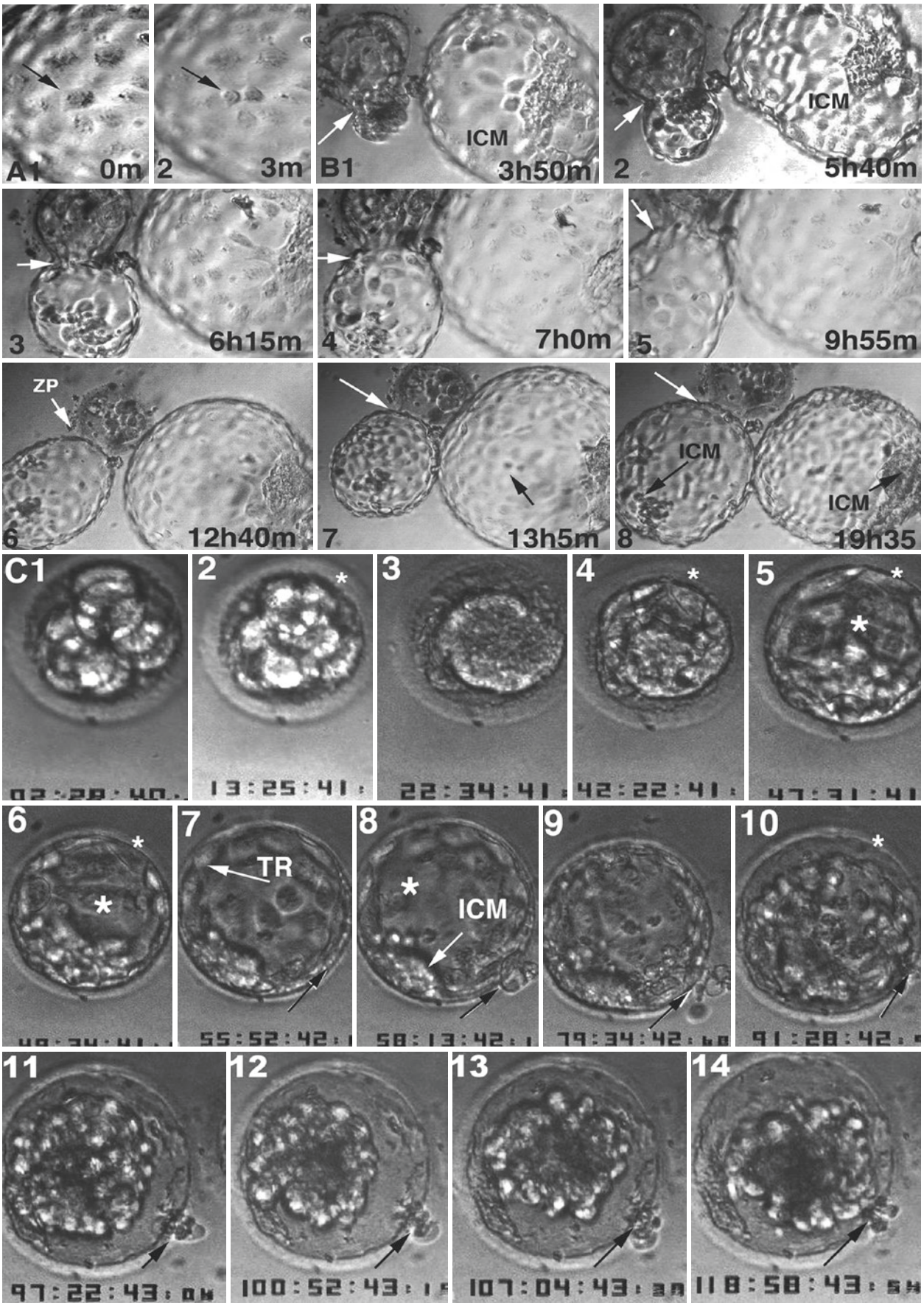
18.17 Lessons from the Introduction of the SCS Through the Walking Egg Program in Resource-Poor/ Developing Countries

During the past few years, experience from establishing a low-cost IVF program, such as the Walking Egg, using the SCS technology in low-resource settings for which the system was initially intended, has been mixed. On the setup and operational side, the experience has been rewarding because it works as designed, but somewhat disappointing with respect to adoption. A lack of commitment, enthusiasm, or willingness to take advantage of a low-cost IVF option has been surprising given that reluctance often comes from the very same sources that have long championed women's reproductive rights, and as a matter of social justice and equality, decried the lack of an affordable treatment for infertile couples claiming availability only for the very wealthy [1–3, 5, 37, 38]. The current reality has been somewhat different, however, where so-called reproductive rights seem to focus mostly on contraception rather than conception that requires medical intervention to create families using assisted reproductive technologies. While considerable opposition from the few established and well-connected high-cost IVF centers was not unexpected, it has become apparent that despite claims for low-cost ART/IVF services from well-intentioned individuals, medical institutions, certain foundations, and government officials, in actuality, they seem unready or unwilling to consider the adoption of a viable option developed specifically to address their often-stated pleas for such advanced reproductive technologies.

Although more than 98% of the population in developing countries is excluded from IVF because of expense or lack of treatment centers, or both, establishing local low-cost programs seems to be a low priority. As former US Secretary of State Colin Powell once said, "A dream doesn't become reality through magic; it takes sweat, determination and hard work." While the "dream" of the Walking Egg Program to provide truly low-cost IVF in developing countries was realized with the SCS system, one lesson learned was that to achieve implementation, a receptive political, governmental, and clinical community are essential preconditions. Fortunately, these preconditions have been met elsewhere where the value of the SCS/Walking Egg Program has been recognized as a viable means of providing affordable IVF. The irony of this experience is that a technology and model for its delivery designed and clinically tested successfully in a high-resource setting(s) specifically for use in low-resource/ developing countries may witness wider application and adoption in developed countries where advanced infertility treatments such as IVF need to become more accessible and affordable as well for their own population.

A second lesson from the Walking Egg experience in so-called developing countries is that the critical shortage of medical doctors, especially those with some familiarity with reproductive medicine, is a major barrier to providing affordable IVF. It is remarkably easy to educate and train laboratory technicians in the SCS method. In contrast, it was much more difficult to train clinicians to perform a well-structured infertility work-up, make an accurate infertility diagnosis where IVF would not be effective without prior intervention (see below), or how to monitor and interpret findings during an IVF cycle, which are elements of treatment largely assumed to be the case in developed countries. In this respect, the streaming video incorporated into the low-cost time-lapse system described here will likely benefit the ongoing training of laboratory personnel owing to access to experts in similar albeit remote laboratories, but is unlikely to have the same utility for an under-trained clinical staff. A third lesson from the Walking Egg experience is that because reproductive healthcare education is virtually nonexistent in parts of Africa, many infertile couples wait too long to seek treatment, mostly due to financial reasons. By this time, 90% of women over age 35 evaluated for infertility have never gotten the message that increasing age is an important prognostic factor in outcome, and when evaluated, are found to have

Fig. 18.5 Panels 1–8 in Fig. **a** demonstrate the level of resolution and fine morphokinetic details of blastocyst expansion and hatching that can be obtained for static imaging or time-lapse microscopy though the glass culture tube of the SCS. The arrow in panels **a**1 and 2 shows that cytokinesis in the trophectoderm is clearly detectable as the single cell in panel 1 becomes two in panel 2. Panels 1–8 in Fig. **b** are from a longer sequence of embryogenesis that begins for two sibling embryos about 4 h (panel **b**1) after the embryo on the right had completely emerged (hatched) from the zona pellucida. While the earlier hatched embryo continued to expand, its slower sibling on the left completed its emergence some 10 h later (panel 7) but by 16 h (panel 8), had expanded to reach a diameter that was similar to its earlier sibling. Both embryos hatched in a continuous fluid motion with no cycles of collapse or expansion of the blastocyst cavity (blastocoel). Panels **c**1–14 show progressive embryogenesis from cleavage (panels 1 and 2), morula (panel 3), cavitation (large asterisk, panel 4), blastocyst expansion with formation of the blastocoel cavity (large asterisk, panels 5, 6, and 8), delineation of the trophectoderm (TR, panel 7), and inner cell mass (ICM, panel 8). However, although apparently normal with respect to morphokinetic activity and morphology during blastocyst formation, hatching failed despite the elaboration of trophectodermal plasma membrane specializations termed "zona breakers" thought to facilitate hatching (black arrow, panels 8–14). The projection detached from the embryo during collapse yet retained independent motility for some hours thereafter within and on the surface of the zona pellucida (panels 11–14). A progressive thinning of the zona pellucida, which is a normal feature of the expansion phase, is evident by comparing thickness from cleavage to expanded blastocyst stages (small asterisk, panels 2, 4, 5, 6, and 10). Time from the start of culture is shown at the bottom of each panel


A1 0m
2 3m
B1 ICM 3h50m
2 ICM 5h40m
3 6h15m
4 7h0m
5 9h55m
ZP
6 12h40m
7 13h5m
ICM
ICM
8 19h35
C1
2 *
3
4 *
5 * *
6 * *
7 TR
8 * ICM
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11
12
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large myomata, uterine polyps, or Asherman disease, which greatly reduces the chances of a successful outcome, whether by the SCS method or with a conventional high-resource IVF laboratory.

Our current experience with the SCS platform and Walking Egg model for its delivery under not atypical conditions for low-resource settings has been successful although challenging. Where advanced infertility treatments are essential to create families, IVF using the SCS can indeed be successful, affordable, and made accessible in both developing and developed countries, but appropriate clinical training in reproductive medicine is a necessary prerequisite for success in low-cost settings and for all settings; educational programs on reproductive health that include the causes of and treatments for infertility are an important component. The ability to proactively address anticipated challenges and productively solve those unanticipated has reinforced the notion that concepts such as the SCS, time-lapse recording, and video streaming for training purposes and embryo selection can be combined with infertility treatment paradigms such as the Walking Egg model in general, and for low-resource settings in particular. For the latter, SCS methodology can be further simplified and adapted to become a truly effective means of treating infertility at low cost. However, the real challenges that now lie ahead in both developed and developing countries may not be technical or with appropriate clinical training, but rather competing interests that promote reluctance or opposition to adoption on the part of established programs and their supporting professional societies in reproductive medicine that can have significant influence on regulatory authorities and philanthropic organizations, including those that champion the reproductive rights and equality for women.

Review Questions

1. What are the basic elements of culture needed to support successful human fertilization and preimplantation embryogenesis in vitro?
2. What modifications could be made to address potential concerns from regulatory authorities about thermal stability for time-lapse microscopy using the simplified culture system?
3. Given the limitations of the simplest iteration of a time-lapse system application for the simplified IVF culture system compared to current commercial systems, what patients may benefit the most with respect to outcome from its use?
4. Why might there be resistance to the introduction of a lower-cost IVF model in both developing and developed countries if treatment can be more affordable and accessible?

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In Vitro Culture of Oocytes and Embryos with Micro-vibration: "Naturalization" of Routine IVF Procedure

Vladimir Isachenko, Karl Sterzik, Gohar Rahimi, Peter Mallmann, and Evgenia Isachenko

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Learning Objectives

- All fertilization steps in nature take place inside the fallopian tube under micro-vibration.
- By routine procedure, embryos are not subjected to ever-changing dynamic processes.
- Limitations of in vitro development of human oocytes/embryos after fertilization.
- It is important to determine whether there is any difference in the viability of embryos after in vitro culture under static and mechanical micro-vibration conditions.
- Evaluation of developmental and "baby-take-home" rates of embryos.

19.1 Introduction

Infertility is the inability of a person to reproduce by natural means. It is usually not the natural state of a healthy adult organism [1]. In that way infertility is a disease, in essence. A reasonable question to propose is whether cell transplantation may be a viable option for the treatment of this disease. In fact, the most effective method of treatment for sterility is harvesting and transplanting embryonic cells (embryo).

Embryo transplantation refers to a step in the process of assisted reproduction in which embryos are placed into the uterus of a female with the intent of establishing a pregnancy. This technique is often used in connection with in vitro fertilization (IVF). In vitro embryo culture is a stage of IVF in which embryos are allowed to grow in an artificial medium [1].

IVF is a process by which an oocyte is fertilized by sperm in vitro. The process involves monitoring and stimulating a woman's ovulation, removing oocytes from the ovaries and letting sperm fertilize them in vitro. The fertilized oocytes are cultured for 2–6 days in a growth medium and are then transplanted into the uterus with the intention of establishing a successful pregnancy. It is a technique of assisted reproductive technology for treatment of sterility [1].

All fertilization steps in nature (receiving the ovulated oocytes, fertilization of oocytes, and embryonic development with subsequent transport to the uterus) take place inside the fallopian tube. The tubal mucosa is arranged as longitudinal folds and consists of a single layer of cuboidal or columnar epithelium. The major cell types of this epithelium are secretory and ciliated cells [2], whose cilia have a vibration [3] (beat). Some investigations have shown an increase in ciliary beat frequency to 5.8 ± 0.3 Hz in the fimbrial section of the tube during the secretory phase [2] compared with the proliferative phase (4.9 ± 0.2 Hz).

What is known about micro-vibration in general? Vibration is a natural phenomenon that refers to mechanical oscillations about an equilibrium point. Since life began, the Earth has subjected all living things to a natural pulsation frequency. This natural phenomenon was predicted in 1952 [4] and named the global electromagnetic resonance phenomenon or Schumann resonances. Schumann resonances are quasi-standing electromagnetic waves that exist in the Earth's "electromagnetic" cavity (the space between the surface of the Earth and the ionosphere). Schumann resonances are the principal background in the electromagnetic spectrum between 3 and 69 Hz and appear as distinct peaks at extremely low frequencies of around 7.83 (strongest), 14, 21, 27, 39, and 45 (weakest) Hz. In daily life, this vibration could be "desirable" (e.g., musical instruments) but more often is undesirable (wasting energy and creating unwanted sound – noise).

The stimulating effect of vibration on living systems is well known and plays a relevant role in mechanical transduction, which is essential for the survival of both cells and higher organisms.

A retrospective analysis was done using a cohort of patients who received intracellular sperm injections (ICSI) at a German private fertility center about 2 years ago [5]. In vitro culture was performed either in a static environment with single oocyte/embryo culture (n = 291 patients) or under micro-vibration and group culture (n = 244 patients). In the static group, oocytes/embryos were cultured individually, while in the micro-vibration all the oocytes were cultured together and up to four embryos were cultured in the drop with a three-dimensional vibration of 56 Hz for 5 s/60 min. Authors observed a significant increase in the fertilization rate in oocytes cultured in groups and under micro-vibration conditions compared to oocytes cultured individually in a static culture (82% vs. 78%); there was also an increase in the implantation rate under these conditions (42% vs. 35%). At the same time, authors noted that the clinical pregnancy rate showed a tendency to be higher in the micro-vibration group but it did not reach significance (47% vs. 43%) [5].

The aim of our investigations was to determine whether there was any difference in the viability of embryos after in vitro culture under static and mechanical micro-vibration conditions.

19.2 Materials and Methods

The authors confirm that all ongoing and related trials for this drug/intervention are registered. Clinical trial registration: ISRCTN13773904 "In vitro culture and transfer of human embryos."

The current work was performed from January 2011 to December 2015 at a private medical center (Endokrinologikum Ulm, Praxisklinik Frauenstraße, Ulm, Germany). Permission was granted by the Ethical Commissions of Medical Faculties of the University of Ulm, Germany (permission 321/10-UBB/bal. from 12.11.2011), and the University of Cologne, Germany (permission 13–147 from 20.11.2013), for the in vitro culture of embryos under mechanical micro-vibration. In vitro culture of embryos of

each odd couple was performed in accordance with routine methodology in a static system (without micro-vibration). Each even couple was offered the choice of the in vitro culture of oocytes and embryos according to the standard routine or with mechanical agitation (micro-vibration) until transplantation. Written informed consent was obtained from all the participating couples. Eight participants did not agree to the in vitro culture of their embryos with micro-vibration, and their embryos were cultured in a static "traditional" system.

All patients with infertility were stimulated for IVF cycle or intracytoplasmic spermatozoa injection (ICSI) cycle with triptorelin (Decapeptyl®, Ferring, Kiel, Germany) and recombinant follicle-stimulating hormone (FSH; Puregon®, MSD Sharp & Dohme GmbH, Haar, Germany; or Menogon®, Merck Serono GmbH, Darmstadt, Germany; or Gonal-f®, Merck Serono) according to the "short" protocol. Ovulation was induced by the administration of 5000 IU of human chorionic gonadotropin (hCG; Brevactid®, Ferring), and oocytes were retrieved 34–36 h later and inseminated with the partner's sperm through conventional IVF and ICSI techniques.

Patients were alternately assigned to the two embryo culture groups. Only two or three embryos per patient were cultured, as according to German law no more than three pronuclear oocytes/embryos from one patient (usually two) can be cultured in vitro and all cultured oocytes/embryos must be later transferred to the patient independently of the developmental rate of these embryos.

Oocytes for the culture of pronuclear embryos were obtained from 4436 patients who provided informed consent (aged 26–44 years, median age 32.8). Pronuclear embryos (two or three per patient) were cultured in vitro under two different conditions: Group 1 ($n = 4821$), without mechanical agitation of the culture medium (standard routine conditions), and Group 2 ($n = 4803$), with mechanical agitation (44 Hz delivered over 5 s once every hour) and acceleration (660 mV/g at 3.3 V: $X = \pm 1.0$ g, $Y = \pm 0.7$ g, $Z = \pm 0.15$ g).

Mechanical agitation was achieved using the developed device Viboviduct 1500 (SimSoTec GmbH, Cologne, Germany). Before using the device, it was calibrated by measurement of vibration with special device PCE-VT 2700 (PCE Instruments UK Ltd., Southampton, UK). Viboviduct 1500 generates micro-vibrations by use of a special electric motor with low electromagnetic noise. The generated vibrations are forwarded directly to the plate containing affixed Petri dishes with embryos. Harmful high frequencies are dampened and smoothed by the intelligent control software developed on the microprocessor. The control software monitors the motor movements. The device is designed and developed for use in a CO_2 incubator.

Embryo development rates were determined on the day of transfer (Day 2, Day 3, or Day 5). The embryos were cultured in 50 µl of culture medium (Sage, Los Angeles, CA, USA) under mineral oil (Sigma-Aldrich, St. Louis, MO, USA) for their transfer.

The embryos were graded on Day 2 and Day 3, as described by Steer et al. [6], as follows: Grade A, equal sized symmetrical blastomeres; Grade B, uneven blastomeres with <10% fragmentation; Grade C, 10–50% blastomeric fragmentation; and Grade D, > 50% blastomeric fragmentation. Day 5 embryos were graded according to Veeck and Zaninovic [7].

Embryo transfer was performed on Day 2, Day 3, or Day 5 after retrieval of oocytes. Pregnancy was defined as an increase in hCG serum concentration (20 IU/L), which was determined 11 and 13–15 days after embryo transfer. Clinical pregnancy was recorded when the fetal sac was visualized on an ultrasound at gestational weeks seven to eight.

19.3 Statistical Analysis

Quality of embryos, amount of transferred embryos, amount of morphologically ideal developed embryos (Grade A and B), amount of sacs, pregnancy outcome (ongoing pregnancy, abortion, abrasion, biochemical pregnancy), and baby-take-home rate (the number of live births per number of IVF/ICSI treatments (cycles) (expressed as percentages)) were evaluated by analysis of variance (ANOVA). Various characteristics were summarized by mean and standard deviation (SD) within groups. The level of statistical significance was set at $P < 0.05$. Clinical pregnancy rates were analyzed by ANOVA for categorical variables using the CATMOD Procedure of SAS Institute Inc. (Cary, NC, USA). Comparisons between age groups were performed by pairwise contrasts and Bonferroni-Holm adjustment for multiple comparisons using the MULTTEST procedure of SAS.

19.4 Results

The mean numbers of transferred embryos per patient for the static group and the micro-vibration group were 2.17 ± 0.32 and 2.17 ± 0.36, respectively.

For groups ≤29 years, 30–34 years, 35–39 years, and ≥40 years, the following rates of high-quality embryos without fragmentation were observed (2–4 blastomeres on Day 2; 6–8 blastomeres and compacting morula on Day 3; blastocyst, expanded and hatching blastocyst on Day 5) (static vs. vibration, respectively): 65.2% vs. 70.8%, 44.3% vs. 69.3%, 67.% vs. 76.4% (for statistic significant differences between respective rates in these three groups $P < 0.05$), and 67% vs. 66% ($P > 0.1$) (Tables 19.1 and 19.2).

The following baby-take-home rate was detected for groups ≤29 years, 30–34 years, 35–39 years, and ≥40 years, respectively (static vs. vibration): 30% vs. 31% ($P > 0.1$, increasing only on the level of tendency), 28% vs. 37%, 23% vs. 29%, and 9% vs. 15% (differences between respective rates in these three groups with $P < 0.05$) (Table 19.3).

Table 19.1 High-quality embryos in different age groups just before transplantation: in vitro culture in static system

Quality of embryos	Age of patients			
	≤29 years	30–34 years	35–39 years	≥40 years
Day 2				
2A	26%	13%	12%	6%
4A	35%	25%	49%	59%
Total high-quality embryos [*n* (%)]	18 (61%)	23 (38%)	110 (61%)	71 (65%)
Day 3				
6A	17%	5%	13%	15%
8A	46%	29%	48%	48%
8A compacting	9%	6%	9%	9%
Total high-quality embryos [*n* (%)]	55 (64%)	98 (40%)	524 (70%)	393 (72%)
Day 5				
EB	14%	14%	17%	24%
B3	1%	1%	1%	2%
B2	18%	1%	19%	11%
B1	27%	24%	24%	20%
B1h	9%	15%	8%	6%
Total high-quality embryos [*n* (%)]	264 (69%)	633 (55%)	568 (69%)	292 (63%)*

(2A) 2 blastomeres, no fragmentation; (4A) 4 blastomeres, no fragmentation; (6A) 6 blastomeres, no fragmentation; (8A) 8 blastomeres, no fragmentation; (8A compacting) 8 blastomeres, beginning of compacting; (EB) early blastocyst; (B3) blastocyst with small blastocoel; (B2) blastocyst; (B1) expanded blastocyst; (B1h) fully expanded or hatching blastocyst
Only underlined rates (percentages) were compared (Table 19.1 vs. Table 19.2). Only rates (percentages) marked with (*) have no significant difference ($P > 0.1$) (Table 19.1 vs. Table 19.2)
The rest of respective rates (percentages) are significantly different ($P < 0.05$) (Table 19.1 vs. Table 19.2)

19.5 Discussion

There was no significant difference between the quality of embryos from some groups (e.g., in group ≥40 years), but the baby-take-home rate was significantly different. We believe the subjectivity of the process by which embryos are evaluated may affect the results, as interpretations vary depending on the embryologist performing the evaluation. However, the number of babies born is an absolutely objective rate that is free from subjectivity and we have drawn our conclusions based on this parameter.

The conditions of embryonic development in vivo include ciliary beating from the moment ovulation begins, fertilization, and during embryo transport via the oviduct to the uterus [8]. "Ciliary" refers to the cilia, which is Latin for vibrating hairs. Baseline cilia beating frequencies have been reported to vary widely between individuals in the range of 5–20 Hz [9, 10]. Ciliary beat has the following characteristics: (i) its rate is remarkably uniform [1]; and (ii) the beat of a particular cilium and its adjacent cilium appears to be well coordinated and a definite metachronal wave is established [11]. This metachronism is defined as coordinated oscillation including a definite phasing of micro-vibration between the cilia of a single cell and a definite phasing of this vibration between the cilia of adjacent cells. The fluid that surrounds the cilia and forms a blanket above the tips of the cilia is a suspension of mucus [12].

The positive effect of pulsatile mechanical micro-vibration for the cytoplasmic maturation of in vitro mature pig oocytes was described in a previous study [13]. These authors subjected cumulus–oocyte complexes cultured in micro-drops to pulsatile mechanical vibration at a frequency of 20 Hz with acceleration (660 mV/g at 3.3 V: $X = \pm 1.0$ g, $Y = \pm 0.7$ g, $Z = \pm 0.15$ g; instruction of manufacturer). During in vitro maturation, vibration did not affect the proportion of oocytes reaching the metaphase II stage. However, blastocyst formation rates after the activation of oocytes exposed to vibration were significantly higher than those obtained for oocytes matured without mechanical vibration (27% vs. 12% and 26 vs. 15%, respectively, for the 5 s and 10 s pulses).

Table 19.2 High-quality embryos in different age groups just before transplantation: in vitro culture with micro-vibration

Quality of embryos	Age of patient			
	≤29 years	30–34 years	35–39 years	≥40 years
Day 2				
2A	22%	16%	20%	20%
4A	44%	48%	51%	38%
Total high-quality embryos [*n* (%)]	22 (66%)	72 (64%)	222 (71%)	97 (58%)
Day 3				
6A	9%	11%	12%	11%
8A	55%	48%	54%	52%
8A compacting	6%	7%	12%	14%
Total high-quality embryos [*n* (%)]	106 (70%)	335 (66%)	448 (78%)	263 (77%)
Day 5				
EB	17%	17%	19%	20%
B3	1%	4%	0%	0%
B2	14%	9%	17%	13%
B1	28%	26%	33%	22%
B1h	17%	21%	9%	7%
Total high-quality embryos [*n* (%)]	263 (77%)	653 (77%)	804 (78%)	238 (62%)*

(2A) 2 blastomeres, no fragmentation; (4A) 4 blastomeres, no fragmentation; (6A) 6 blastomeres, no fragmentation; (8A) 8 blastomeres, no fragmentation; (8A compacting) 8 blastomeres, beginning of compacting; (EB) early blastocyst; (B3) blastocyst with small blastocoel; (B2) blastocyst; (B1) expanded blastocyst; (B1h) fully expanded or hatching blastocyst
Only underlined rates (percentages) were compared (Table 19.2 vs. Table 19.1). Only rates (percentages) marked with (*) have no significant difference ($P > 0.1$) (Table 19.2 vs. Table 19.1)
The rest of respective rates (percentages) are significantly different ($P < 0.05$) (Table 19.1 vs. Table 19.1)

In medicine, the embryonic development rates and clinical results were compared between a static culture group (n = 159 cycles) and a micro-vibration culture group (n = 166 cycles) in poor responders. A micro-vibrator was set at a frequency of 42 Hz for 5 s/60 min duration of embryo development [14]. In poor responders, the embryonic development rate was improved to a limited extent under the micro-vibration culture conditions, but the clinical results were significantly improved[15].

It should be noted that the vibration at a frequency of 44 Hz and the acceleration described above are the parameters of movement of the plate on which Petri dishes with culture medium and embryos are located. The study laboratory's observations on bovine oocytes have shown that the amplitude of vibration of embryos as well as acceleration of cells is lower than the rates with which the plate is vibrating (data not shown). This fact is due to the inertness of oocytes suspended in a liquid environment; vibration is drastically suppressed by the culture medium and is dependent on the composition and volume of this medium. Parameters of the "real" vibration of embryos are calculated mathematically and make up 33 Hz. It is necessary to emphasize that the observations in vivo and published rate of vibration of cilia are different because the embryo is "swimming." Therefore, knowing the rate of embryonic development is a criterion for adjusting the frequency of the apparatus vibration.

The acceleration accompanying any movement ($\mathbf{a} = \mathbf{r}\boldsymbol{\omega}^2$ and $\boldsymbol{\omega} = 2\pi\mathbf{f}$, (**a**) acceleration, (**r**) radius of movement, (**f**) frequency) is crucially important for biological objects. The maximal rate of acceleration is achieved at a maximal frequency of 50 Hz. That is why it would not be correct to use the "optimal" rate of vibration frequency established for an apparatus. It is also important to note that apparatus with the same frequency may have a different acceleration rate that should be taken into consideration.

In conclusion, in vitro culture of embryos under micro-vibration (mimicking conditions in nature whereby oviductal fluid is mechanically agitated by the epithelial cilia) significantly increases the baby-take-home rate for patients 30 years of age and older.

Table 19.3 Number of formed sacs and "baby-take-home" rate after transplantation of developed and transferred embryos in different age groups: in vitro culture in static system and with micro-vibration

	In vitro culture in static system				In vitro culture with micro-vibration			
	≤29 years (496 embryos from 250 patients)	30–34 years (1457 embryos from 715 patients)	35–39 years (1751 embryos from 804 patients)	>40 years (1117 embryos from 457 patients)	≤29 years (527 embryos from 277 patients)	30–34 years (1469 embryos from 690 patients)	35–39 years (1911 embryos from 850 patients)	≥40 years (893 embryos from 393 patients)
Number of sacs [*n* (%)]	149 (30%)	525 (36%)	595 (34%)*	68 (16%)	268 (51%)	646 (44%)	671 (35%)*	286 (32%)
Singleton [*n* (%)]	88 (59%)	315 (60%)	327 (55%)	178 (76%)	145 (54%)	323 (50%)	463 (69%)	254 (89%)
Twins [*n* (%)]	61 (41%)	204 (39%)	268 (45%)	43 (24%)	123 (46%)	200 (48%)	201 (30%)	32 (11%)
Triplets [*n* (%)]	0 (0%)*	6 (1%)*	0 (0%)*	0 (0%)*	0 (0%)*	13 (2%)*	7 (1%)*	0 (0%)*
"Baby-take-home" rate [*n* (%)]	149 (30%)*	408 (28%)	403 (23%)	100 (9%)	163 (31%)*	543 (37%)	556 (29%)	134 (15%)

Baby-take-home rate notes the number of life births per number of IVF/ICSI treatments (cycles) in percent
Only respective rates (percentages) marked with (*) have no significant difference ($P > 0.1$)
The rest of respective rates (percentages) are significantly different ($P < 0.05$)

Review Questions

1. Is the routine procedure of in vitro culture of oocytes/embryos mimicking conditions in nature?
2. What are the limitations of in vitro development of oocytes/embryos?
3. What is the source of micro-vibration in the testing equipment?
4. Is using of micro-vibration beneficial by in vitro culture of oocytes and embryos?

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Microfluidics for Gamete Manipulation and Embryo Culture

Peng Yuan, Liying Yan, and Gary D. Smith

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Learning Objectives

This chapter aims at informing the reader of the following:

- Potential uses of microfluidics in gamete and embryo isolation, manipulation, and analysis
- State of the art of microfluidics and gamete and embryo culture in numerous species
- Practical reasoning for use of microfluidics for gametes and embryos
- Futuristic view of how and where microfluidics might be used in human ART

20.1 Introduction

20.1.1 Introduction of Human ARTs (Assisted Reproductive Technologies)

Human ARTs refer to all treatments or procedures that include the in vitro handling of human oocytes, sperm, and embryos, for the purpose of establishing a pregnancy. This includes, but is not limited to, in vitro fertilization and embryo transfer, gamete and embryo cryopreservation, oocyte and embryo donation, and gestational surrogacy [1]. Since 1978, the world's first "test-tube" baby—Louise Brown—was born via in vitro fertilization (IVF), and the past three to four decades have seen a rapid evolution in human ARTs. Advances in ARTs, including the use of donor gametes and intracytoplasmic sperm injection (ICSI), have led to increased numbers of infertile couples being treated with ARTs in recent years.

20.1.2 Present Shortcomings of ARTs

The continuous refinement of ARTs in the past decade has mainly been the modification of medium components, concentrations, and the addition of new elements at specific time points. While there have been many advances in in vitro manipulation of embryos in regard to the efficiency and the quality, these embryos are still suboptimal compared to their in vivo counterparts [2]. These differences in the quality of embryos are believed to be partially associated with stresses that are imposed on the oocyte/embryo during the ART procedures. It begins as early as the time of transvaginal oocyte retrieval, as small temperature fluctuations can have significant effects on oocyte quality, which can negatively influence the following IVF procedure [3]. Once the oocyte enters the laboratory, there can be as many as 20 manipulations that potentially impose stress (change in pH, osmolality, temperature, and light) on the oocyte/embryo [4, 5]. Such continued stresses have their adverse effects directly on the gametes/embryos, which can be detrimental to subsequent embryo development potential. Most research, with aims of reducing these types of stresses during ART procedures, have focused on the media modulation rather than finding systems that could reduce the stress introduced by the manipulation. Furthermore, the physical microenvironment and chemical/physical aspects of that microenvironment that support oocyte short-term culture, maturation, fertilization, and embryo culture require enhanced study and refinement.

Despite the wide application of ARTs around the world, two major problems remain; low implantation rates and high multiple gestation rates. As a significant cause of physical, emotional, and financial distress for women undergoing infertility treatments, pregnancy failures happened to approximately two out of three couples during ART cycles [6]. A reasonable explanation for the high rate of failure in ART cycles is the inability to determine the embryos with highest developmental and/or implantation potential. During the past three to four decades, morphology and cleavage rates have been the major methods used for embryo assessment and selection for uterine transfer in clinics. Currently, individual embryo chromosome normalcy is being assessed with the goal of improving implantation rates. Other embryo-specific biomarkers could also assist in selecting embryos with the greatest development and implantation potential. Besides the stresses on the oocyte/embryo from manipulation in vitro, there are also human factors associated with ARTs, which can result in losses of oocytes/embryos at each manipulation step. During the treatment of human infertility, where costs of embryos are high, loss of any oocyte/embryo, at any step, could be potentially detrimental to the success of patients in family building, the infertility clinic success, and good standing.

20.1.3 Practical Logic of Why, and How, Microfluidics May Be Applied to Human ART

One possible solution to reduce these stresses on gametes/embryos may be the application of micro- and nanotechnologies to laboratory ARTs, particularly microfluidic technologies. In relation to cell biology, microfluidic technologies first emerged in the late 1980s and early 1990s with initial reports of individual cell fusion in a silicone fluidic device and transport of animal cells [7, 8].

Microfluidics is a fast growing, highly interdisciplinary subject, covering physics, engineering, chemistry, and biology. From a technology perspective, microfluidics refers to microscale devices that can precisely manipulate fluids. Microfluidic technologies were first developed by the semiconductor industry and later expanded by the microelectromechanical systems (MEMS) field. These devices could be applied to biology studies to facilitate complex assays through (1) minimizing the sample volume needed, (2) reducing the reagent consumption and maximizing information collected from scarce samples, (3) expanding screening scope and capacity of batch sample processing, and (4) providing researchers precise control and expectedness of the spatial-temporal dynamics of the microenvironment.

In order to solve problems in biology and clinical research, biomedical engineers are devoted to developing comprehensive microfluidic technologies. Application of microfluidics

to embryo manipulation in vitro may alleviate some of the drawbacks that traditional microdrop-culture places on embryo culture. Microfluidic technologies are promising in human ART, providing the capability to manipulate gametes and embryos in considerably small volumes of culture medium, in a nano- to microliter range. The physical characteristics of liquids in microenvironments have been well studied; thus, it may be advantageous for biologists and engineers to work together in developing microfluidic devices for manipulating gametes or embryos in microenvironments.

These microfluidic platforms could precisely simulate in vivo microenvironments in relation to fluid volume/cell mass ratios, as well as reduce the cost of reagents. Furthermore, through reducing the sample volume, beneficial paracrine and autocrine factors that promote embryo development may be concentrated around the embryos [9–12]. Microfluidic devices may also improve the fertilization rate in vitro by allowing spermatozoa and oocytes to interact in a more in vivo-like environment, whereby only a few motile spermatozoa flow past the oocyte and participate in the insemination/fertilization process. More important, microfluidic devices can facilitate the study of dynamic processes of embryo development. With the application of microfluidic technologies in ART laboratory platforms, the stress imposed on embryos may be reduced or alleviated, since the embryo would grow in an environment where suitable osmolality, temperature, and pH are maintained. Another advantage of microfluidics is the automated control of media changes and flow rates. Thus, utilizing microfluidic devices would not only eliminate mechanical/biological stress on gametes and embryos, but would also remove potential human error. Finally, microfluidic devices would permit gametes and embryos to grow in dynamic microenvironments that more closely imitate the in vivo development environment, with automatic experimental manipulations. Numerous interdisciplinary groups are actively researching the potential benefits of microfluidic gamete/embryo manipulation and culture.

20.2 State-of-the-Art Research of Microfluidics and ART in Rodents, Domestic Animals, Nonhuman Primates, and Humans

20.2.1 Microfluidics for Male Gametes

20.2.1.1 Microfluidics for Semen Analysis

Despite multiple and evolving approaches to evaluating and diagnosing male factor infertility, routine semen analysis is often the first and most important test performed. Total concentration and motile percentage of spermatozoa are two major factors to be addressed in semen quality assessment. When comparing ejaculates, significant trends are apparent in relation to total sperm concentrations, motile sperm percentages, and morphologies between fertile and infertile males [13]. Throughout the current decade, the microfluidic technologies have been evaluated in the clinical context, including human semen analysis [14–17].

As sperm are motile cells capable of forward movement, microchannel devices may offer a qualitative assessment of sperm motility and ability to separate motile sperm from nonmotile sperm [14], and they also enable quantification of the motile sperm content of human ejaculates. These devices can also couple microfluidic engineering with fluorescence labeling of live sperm and use a microfluorometer to quantify motile sperm [17, 18]. A novel technology was presented, in which the device is lightweight and disposable, making it appropriate for testing human ejaculate outside of the clinical laboratory. The addition of fluorescence labeling of sperm and a microfluorometer to the microfluidic design described has enabled quantification of the motile sperm component of a semen specimen; the signal recorded by the microfluorometer is hypothesized to correlate with the motile concentration of a given semen sample. Other components of the ejaculate, including dead, immotile, or nonsperm cells, or particulate matter, cannot contribute to the signal. Results demonstrated a significant correlation of signal strength with various computer-assisted semen analysis (CASA) parameters, including total and progressive motile sperm concentration, as well as sperm concentration. This microfluidic device was small and fully integrated, and it may find application outside of the traditional laboratory setting, perhaps further opening the door to reliable male subfertility screening at home.

Another configuration of the microfluidic device for assessing the total number of sperm and the percentage of motile sperm is based on the principles of the sperm's random swimming and sedimentation [19]. This platform can also provide information about numbers of motile and immotile sperm, thus assessing both total sperm concentration and motile sperm percentage simultaneously. This microfluidic device is suitable for sperm quality analysis without the aid of expensive microscopes.

Lastly, and very promising, there is a microfluidic device that can discriminate between normal, apoptotic, and necrotic cells without any labeling [20]. This is quite interesting in that apoptosis is directly related to subsequent DNA fragmentation. While not yet reported to have been applied to semen or washed sperm, if feasible, this device has the potential to separate the different sperm population subtypes. Isolating normal from apoptotic spermatozoa has the potential for increasing not only in vivo and in vitro fertilization rates but also ongoing pregnancy rates.

20.2.1.2 Microfluidics for Sperm Functional Tests

Semen analysis is often the first step performed to investigate semen quality. Good semen analysis results do not always imply that the spermatozoa are functioning properly. To further investigate the ability of spermatozoa to fertilize an oocyte, sperm function tests have been established [21–23]. Several sperm function tests exist today, which can be divided into tests that investigate the functioning of the spermatozoa directly by interaction assays and indirectly by biochemical

assays [24, 25]. For example, sperm characteristics such as the acrosome state and DNA integrity have shown significant impact on ART outcome [26, 27].

Advanced sperm selection techniques focus on additional sperm characteristics such as DNA integrity, apoptosis, membrane maturation, and ultramorphology. Ideally, ART procedures are performed using viable, morphologically normal spermatozoa with a high DNA integrity and intact plasma and acrosomal membranes. Especially, ICSI treatments are dependent on accurate selection, since only one spermatozoon is used during each procedure. However, most selection procedures and sperm quality-related studies rely on population-based approaches, which are not applicable (yet) on the single-cell level. The lack of comprehensive single-cell information could explain why sperm selection based on functional characteristics, which showed correlation with fertilization potential in population-based studies, fails to achieve clinical relevance in ART procedures. Wagenaar and his colleagues report the design of a simple polydimethylsiloxane (PDMS) microfluidic platform in which sperm cells were entrapped noninvasively by hydrodynamics [28]. Sperm characteristics such as the cell viability, the acrosome state, and chromosomal content were studied on individual entrapped sperm cells. This platform allows (noninvasive) analysis on the single-cell level and has the potential to be a versatile tool for selection applications or fundamental studies on spermatozoa. However, the current analysis techniques depend on the use of fluorescent labels, which limits the use of entrapped sperm cells after analysis. Therefore, future single-cell analysis must be performed label-free and noninvasively to retain good sperm quality for ART applications after analysis.

20.2.1.3 Microfluidics for Sperm Sorting

One in six couples of reproductive age worldwide is affected by some form of infertility, and male factor plays a role in approximately 50% of the cases of subfertile couples [29, 30]. Various disorders caused by spermatogenesis abnormalities, hormonal disturbances, and physical and psychological problems can cause male factor infertility. However, in the clinical context, it is often difficult to determine the etiology for each individual, although infertile male patients can have low sperm production and poor sperm quality. Assisted reproductive technologies provide a set of powerful techniques to assist couples facing infertility issues. Intracytoplasmic sperm injection (ICSI) is a method, which currently serves as the standard for managing male-factor infertility, that can increase fertilization rates compared to other technologies [31, 32]. Intracytoplasmic sperm injection enables fertilization of an egg with one sperm selected from ejaculated/surgically retrieved spermatozoa. Therefore, sperm quality parameters, including motility, morphology, viability, DNA integrity, apoptosis, and maturity, are important determinants of successful ICSI and ART outcomes [33–35].

The ICSI procedure starts with adding a fraction of the sperm suspension to a mineral oil-covered microdroplet [36], then the sperm to be injected is selected within a microinjection pipette. Sperm selection during the ICSI procedure can be a laborious and time-consuming process. Selection of sperm with good quality is crucial to successful ICSI because fertilization of the oocyte, and ultimately a viable birth, depends largely on sperm viability [37]. Thus, technologies to facilitate identification and selection of healthy sperm can greatly improve the success rate of ICSI, IVF, and other ARTs.

Selection of appropriate sperm for IVF and ICSI is generally based on sperm motility, because motile sperm are viable sperm. The swim-up and density gradient-based centrifugation methods are major traditional sperm-selection techniques [38, 39]. The swim-up method enables separation of motile sperm from nonmotile sperm and semen; however, the technique can be problematic in regard to low yield of motile sperm. The density gradient-based centrifugation can select sperm based on their density; however, density gradient centrifugation has also been shown to potentially have negative effects on sperm viability and give rise to sperm DNA fragmentation [40]. Moreover, these traditional methodologies are less useful for samples with low sperm counts and/or reduced sperm motility [41, 42]. Thus, the limits of traditional methodologies present an opportunity for developing new methods of sperm selection.

The advent of novel micro- and nanodevices presents promising solutions to a wide range of clinical problems. Recently, studies have employed microfluidics to sperm sorting, representing significant progress for improving ART [14]. One of the advantages of utilizing microfluidic devices in ARTs is one-step sorting without the need for centrifugation. Eliminating the centrifugation step minimizes the exposure of sperm to reactive oxygen species (ROS) and further DNA fragmentation [43]. Inspired by in vivo natural sperm sorting mechanisms where vaginal mucus becomes less viscous to form microchannels to guide sperm toward the egg, a microfluidic device was presented that efficiently sorts healthy, motile, and morphologically normal sperm without centrifugation [44]. The design consists of a single channel and a retrieval chamber with a polycarbonate membrane filter (Fig. 20.1). This method resulted in a higher percentage of sorted sperm with retained DNA integrity and fewer reactive oxygen species compared to samples sorted using the conventional swim-up method. Lately, a clinically applicable microfluidic device was described that isolates sperm based on the progressive motility in 500 parallel microchannels and represents a one-step procedure for sperm selection with high motility and high DNA integrity sperm [45]. This device could process up to 1 ml of semen. These types of device improvements and multiscaling are positive steps forward and will yield microfluidic devices that are more likely to find acceptance in clinical laboratories of the future.

20.2.2 Microfluidics for Female Gametes

20.2.2.1 Oocyte In Vitro Maturation (IVM)

Among the various steps of gamete manipulation in ARTs, selecting the best oocyte is considered to depend on subjective criteria. It is generally the result of visual estimations of morphological properties of the oocyte itself and surrounding cumulus cells. Application of the microfluidic system in reproductive biology opens new possibilities for the development of

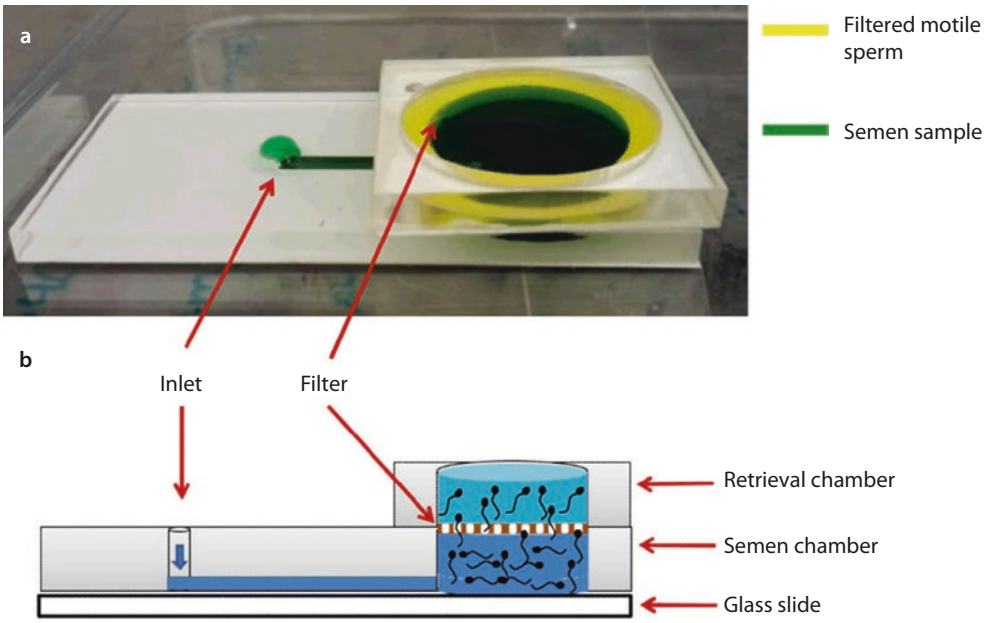

Fig. 20.1 Schematic of macro-microfluidic sperm sorter (MSS) for selection of functional human sperm with higher DNA integrity and fewer reactive oxygen species [44]. **a** The photo of the MSS showing inlet, filter, and two polymethylmethacrylate (PMMA) chambers. The MSS was filled with color dye to enhance contrast. **b** The illustration demonstrates the MSS design and working principle. These images show the comparative size of various filter pores and sperm. The scale bar is 10 μm

techniques available for the assessment of developmental competency manifested by mammalian oocytes and embryos.

While most human ART procedures currently use in vivo matured oocytes, the potential benefits of human oocyte in vitro maturation (IVM) have been appreciated for decades [46–49]. Human oocyte IVM is a technique in human ARTs that has drawn much attention due to its extremely low cost and reduction of ART-associated disorders such as ovarian hyperstimulation syndrome (OHSS) [50]. Oocyte maturation is a complex coordination of cytoplasmic maturation and nuclear maturation that occur in a synchronized manner to embrace successful fertilization. In vivo, mammalian oocytes acquire cytoplasmic maturity and the competence to resume meiosis during follicle and oocyte growth. Thus, proper maturation of the oocyte to metaphase II is a prerequisite for fertilization and preimplantation development. It has been hypothesized that the microfluidic environment may more closely resemble oocyte maturation and/or embryo development conditions in vivo compared to the static microenvironment in a microdrop [51]. Therefore, in the last few decades, much effort has been devoted to improve oocyte maturation. Walters and colleagues demonstrated that porcine oocytes matured in microchannels could achieve the same nuclear maturation as those matured in microdrop. The microchannel has also been used to culture porcine embryos, which resulted in the birth of normal piglets [52]. Hester and coworkers subsequently found that the blastocyst development rate was better when oocyte IVM was performed in microchannels compared to standard static procedures [53]. In 2011, Yuan and colleagues compared two different types of microchannel; each one of these had a subgroup: static or rocking. This study demonstrated the microfluidic well system can successfully mature oocytes individually without compromising oocyte quality or subsequent blastocyst formation after IVF [54]. In 2015, Siavash and colleagues reported a microfluidic device that integrated oocyte trapping and maturation. The device was constructed using standard soft lithography techniques in a way that oocytes were individually monitored and tracked in the devices during their maturation [55]. These studies showed that microfluidic devices have the potential for efficient and healthy culturing and maturation of oocytes.

20.2.2.2 Oocyte Cumulus Cell Removal

Researchers have attempted to develop microfluidic technology for other important procedures during oocyte manipulation, such as cumulus cell removal and/or zona pellucida removal. Cumulus removal is a routinely necessary operation before human oocyte ICSI [56]. There are two conventional methods used to remove the cumulus cells: mechanically by vortexing (in domestic animal IVP) [57] or pipetting with hyaluronidase exposure (human ART) [58]. Microfluidic devices have been adapted to perform cumulus removal. Zeringue and colleagues began studies of microfluidics for cumulus cell removal by identifying optimal device designs and materials for cumulus removal in 2000 [59]. Utilizing fluid flow driven manually via attached syringes, exposing oocytes to chemical and physical manipulation, cumulus cells were removed from bovine oocytes after IVF. Following these pioneering experiments, Zeringue and coworkers further explored the impact of two cumulus cell removal techniques on embryo production: traditional (vortexing or chemically) versus a microchannel device [60]. Results suggested that microfluidics could effectively alleviate the pressure imposed on oocytes during the cumulus removal procedure.

20.2.3 Microfluidics for In Vitro Insemination

20.2.3.1 Microfluidics for Conventional In Vitro Insemination

Following successful isolation of gametes, the next procedure is in vitro insemination. The process of in vitro insemination involves coincubation of oocytes with an appropriate number of

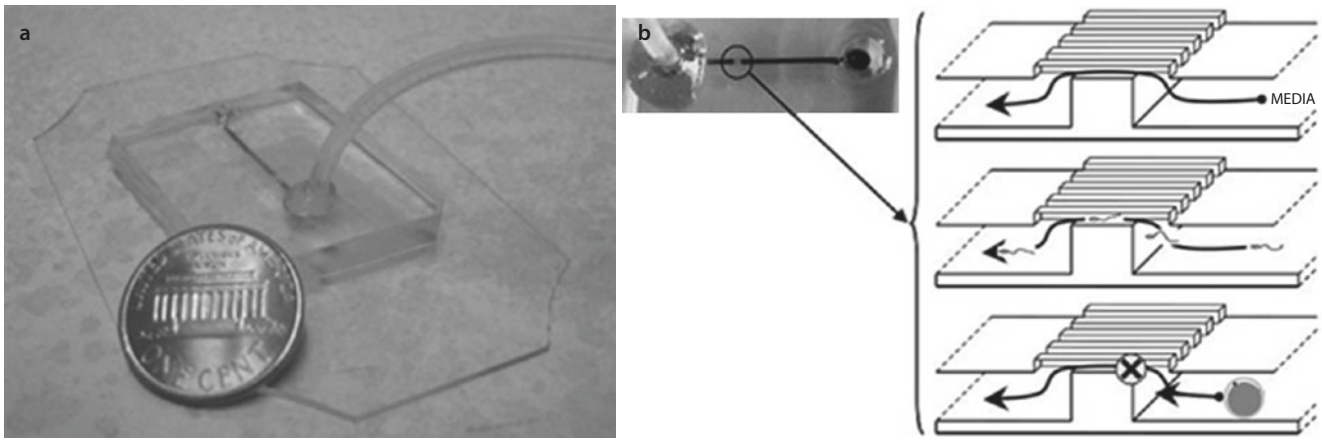

Fig. 20.2 A modified microfluidic platform used to perform fertilization of mouse oocytes [61]. **a** Actual device size in comparison to a US penny. Microchannel was filled with blue dye. **b** Schematic angle view. The three-dimensional construct of the channel prevents movement of the oocyte beyond the grate, but allows for flow of medium and sperm through the channel

motile sperm in an easily controllable and physiologically suitable environment. Present IVF techniques basically utilize culture dishes, culture tubes, or microdrops covered by mineral oil to provide a proper sperm concentration for in vitro insemination; the total number of sperm needed for fertilization is dependent on system volume. Media volumes may range from 20 μl to 1 ml [61]. Such techniques sometimes are inadequate for conventional insemination in an IVF cycle. Alternatively, ICSI is a complementary treatment of male factor infertility [62, 63].

Microfluidics may be particularly suitable to conventional insemination in IVF cycles, for a number of reasons. Firstly, the microenvironment of a microchannel more closely mimics in vivo fertilization microenvironment than a culture dish or microdrop. Secondly, the application of microfluidic channels allows for nonturbulent bathing of gametes with fresh media throughout insemination and coincubation. Sperm-oocyte interactions occur in such an active environment, rather than the static conditions present in a culture dish or droplet. In addition, via laminar flow, microfluidics may enable a more predictable delivery of sperm to oocytes during IVF comparing with the random interaction between sperm and oocyte in a culture dish. Lastly, conventional in vitro insemination requires a high number of sperm, which then deplete metabolic substrates and produce potentially harmful waste products in the IVF media; microchannel environments utilize extremely small volumes of media, theoretically achieving insemination concentrations with fewer sperm compared to standard IVF with larger concentrations and volumes. Several microfluidic systems have been developed for in vitro insemination [64, 65]. Suh and colleagues demonstrated successful in vitro fertilization of mouse oocytes with a polydimethylsiloxane (PDMS) microfluidic device which consisted of a series of open slots within a microchannel [61] (Fig. 20.2). Han and colleagues reported a novel microwell-structured microfluidic device that integrates single oocyte trapping, in vitro fertilization, and subsequent embryo culture [61]. This device achieved similar fertilization rates to standard oil-covered drops in Petri dishes while simplifying oocyte handling and manipulation.

20.2.3.2 Microfluidics for Intracytoplasmic Sperm Injection Insemination (ICSI)

Recently, a paper evaluated using microfluidics for ICSI [66]. Matsuura and colleagues demonstrated microfluidics could be used for the porcine ICSI process, which reduced time for manipulation procedures. These proof-of-concept studies in animal models are the beginning. Wagenaar and his colleagues designed a simple PDMS microfluidic platform in which sperm cells were entrapped noninvasively by hydrodynamics [28]. Sperm characteristics such as the cell viability, the acrosome state, and chromosomal content were studied on individual entrapped sperm cells. This platform allows noninvasive analysis at the single-cell level and has the potential to be a versatile tool for selection applications or fundamental studies on spermatozoa. With single sperm trapped and analyzed, it shows promise for single sperm manipulation in ICSI. While human ICSI is widely used, and highly successful, the multiple highly technical steps are labor-intensive and time-consuming, which can lead to human error and individual technician variability. If integration of microfluidics to insemination in conventional IVF and/or ICSI can reduce laboratory human error or variability, and/or reduce the cost while maintaining similar or improved results, microfluidics may have practical utility in future ICSI protocols.

20.2.4 Microfluidics for Embryo Culture

Culture of preimplantation embryos is widely employed every day in IVF centers and consists of one of the key steps in ART protocols. Although it has been used in clinics since the 1970s, protocols for the culture of human preimplantation embryos are neither standardized nor optimized. Current human fertilization in vitro circumvents the female oviduct and manually inseminates, fertilizes, and cultivates embryos in a static fluid environment containing appropriate compounds.

The in vitro environment is important when considering that embryos are held in media for up to 7 days. Standard in vitro protocols use one or two media during embryo culture [67]. This is in contrast to the in vivo situation in which embryos grow in fluid (medium) with a constantly changing environment as they move through the oviduct to the uterus. Some metabolites necessary during one phase of development may not be needed during other stages [67, 68]. One of the first dynamic embryo culture systems was the formulation of sequential media, where each phase of embryo development had specific substrate concentrations [69]. The disadvantages of these systems are the number of times that the operator has to handle embryos, the volume of media, and the potential reduction of positive effects of embryo trophic factors [70]. In such a scenario, microfluidic embryo culture devices may be a better method to deliver sequential media in a dynamic situation. A microfluidic/microchannel system for embryo culture may provide an improved in vivo-mimicking environment to enhance the in vitro development of embryos in the laboratory.

Compared with static fluid environments, microfluidics has the following potential advantages: (1) dynamic culture microenvironment, (2) reduced culture volume, (3) precise control over culture environment, and (4) decreased embryo manipulation, alleviating the stress imposed on embryos.

Thus far, there have been encouraging outcomes with microfluidic embryo culture technology. Several articles have discussed the potential applications and advantages of dynamic environments for embryo culture obtained with microfluidics. The first embryo culture microfluidic device laid the foundation for application with the demonstration of improved blastocyst development, higher survival rates, and fewer degenerated embryos compared to control microdrop culture [71]. This study employed static microchannel culture, allowing for a direct comparison with static microdrop systems. The improved developmental outcome was presumably due to the lower volume and the embryo's enhanced ability to regulate its microenvironment with paracrine and/or autocrine factors. Although the addition of flow conditions is more similar to in vivo environments of embryo culture, it was discovered that simple continuous media perfusion within a microchannel during embryo development was detrimental to embryo development across a range of flow rates accessed [69]. Multiple reasons may relate to these poor outcomes, but principal reasons may be a loss of paracrine and autocrine factors and the inability of embryo self-regulation of its microenvironment due to poor fluid movement control in the microfluidic device.

Subsequently, Dr. Takayama and colleagues reported on a computer-controlled microfluidic platform using vertically moving Braille pins to facilitate integrated pumping of channel-contained fluids through localized deformation of channels made of elastic compounds [72]. This provided a way to precisely regulate fluid flow in microchannels with computer programming, which could be applied to embryo culture platforms. Microfluidic devices allow nano- to microliter volumes of fluids to realize precise and dynamic control of the cell culturing environment. However, evaporation can be especially detrimental to cell/embryo culture in microfluidic chips, where even the slightest amount of evaporation from the small liquid volumes present in microfluidic systems would result in a significant increase in osmolality [73]. Heo and colleagues came up with a solution of evaporation-mediated osmolality shifts with application of a PDMS-parylene-PDMS hybrid membrane. This PDMS-parylene-PDMS hybrid membrane not only greatly suppressed evaporation and osmolality shifts but also possessed the thinness and flexibility necessary to interface with deformation-based microfluidic actuation systems [73]. In addition, it maintained the clarity for optical microscopy and enabled the successful development of single-cell mouse embryos into blastocysts under static conditions, as well as the culture of human endothelial cells under dynamic recirculation of submicroliter volumes of media. These findings shed light on how to overcome evaporation-associated effects in microfluidic cell/embryo cultures.

Afterward, Heo and colleagues developed a dynamic microfunnel embryo culture system that cultured mouse embryos with media refresh in a physiologically pulsatile manner. Compared with embryos cultured in static microdrops and microfunnels, the mouse embryos subjected to microfunnel pulsatile culture had significantly higher number of blastomeres per blastocyst. More importantly, dynamic microfunnel culture significantly improved embryo implantation rates and ongoing pregnancy rates over static culture to levels approaching that of in utero-derived preimplantation embryos [51].

In 2010, Han and colleagues reported an integrated microfluidic device that performed single oocyte trapping, fertilization, and embryo culture of the IVF process [65]. In 2015, Huang and colleagues demonstrated a novel digitalized microfluidic device powered with electrowetting on a dielectric (EWOD). This device cultured an embryo, in a single droplet, in a microfluidic environment to mimic the in vivo environment of embryo development. Embryos developed well and resulted in live births [74]. These results show that the dynamic culture powered with EWOD can manipulate a single droplet containing one mouse embryo and can support culture to the blastocyst stage. Esteves and colleagues also reported that mouse preimplantation embryos could be cultured in microfluidics and studied the impact of fluid flow, embryo density, and media volume [75]. Kieslinger and colleagues have demonstrated that human embryos can be grown in microfluidic devices [76]. These encouraging studies validate the potential for microfluidics being a powerful tool for science research and potentially having clinical applications in the future.

20.2.5 Microfluidics for Embryo Selection

20.2.5.1 Metabolomics

With the application of microfluidics, the ability to culture embryos in very small volumes provides opportunities for research on critical molecular mechanisms of gametogenesis and early embryo development. The ability to automatically

sample and potentially even test small volumes of medium from multiple locations in the device would be critical to permit analyses of embryo metabolism, protein production, viability markers, and other cellular processes during real-time culture of a live oocyte or embryo.

Over time, significant data have accumulated to demonstrate the metabolism of oocytes and preimplantation embryos (mainly) to be a critical functional parameter measured [77–80]. Metabolic biomarkers, including specific pathway substrates (carbohydrates or amino acids, oxygen) and products (ammonium, carbon dioxide, lactate), may provide insight into oocyte/embryo developmental potential. In relation to energy metabolism, it has been demonstrated that noninvasive means of measuring glucose consumption from culture media and lactate production and release into media, as an estimate of embryo glycolytic activity, can be useful in the selection of qualified human embryos [80, 81]. Yet these measurements can be very laborious and not conducive to application in the clinical laboratory workflow.

Concepts of indirect analysis of single embryo health, based on media nutrient level changes, have significant potential as functional assays. To date, there have been at least three reports using microfluidics to perform noninvasive measures of embryo metabolomics. In 2006, O'Donovan and coworkers developed a respirometric microfluidic cartridge to monitor oxygen consumption of two-cell and blastocyst stage mouse embryos [77]. In 2008, Urbanski and colleagues performed proof-of-concept experiments to demonstrate the use of microfluidics to measure embryo metabolism [82]. They designed and tested a microfluidic chip that performed automated metabolic assays to measure glucose, pyruvate, and lactate from submicroliter volumes. In 2012, an automated computer-controlled microfluidic platform for both embryo culture and metabolic analysis on a single integrated device was reported; this system was able to measure time-dependent nutrient consumption by single or multiple (10) live mouse blastocyst-stage embryos, at 20-minute intervals, in real time, with high sensitivity [83].

20.2.5.2 Proteomics/Secretome

The proteome is estimated to consist of over a million proteins, and being derived from the transcriptome, is responsible for cell identity and cell function [84]. Proteomics may be a useful metric on which to base decisions regarding which embryo has the highest developmental potential. Whereas the analysis of the proteome requires cellular extraction and precipitation steps, followed by fractionation (e.g., electrophoretic, chromatographic) and detection (spectroscopic, fluorometric), it is feasible to noninvasively analyze a large number of proteins and peptides secreted by an individual human embryo, the secretome, by sampling the surrounding culture medium [85]. Analysis of the secretome bypasses these earlier preparatory steps, but remains an indirect biomarker of oocyte or embryo physiology. Katz-Jaffe and coworkers identified an 8.5-kDa protein whose abundance increased only in the secretome of developing blastocysts, indicating a potential relationship between this protein and developmental potential [86]. Such analyses can be done without microfluidics, yet integration of microfluidic platforms may be advantageous.

Microfluidic devices are ideally suited to profiling single blastocyst secretomes in culture media, and could easily accommodate cell lysates as well (if desired). Proof-of-concept microfluidic devices have been employed for on-chip cell lysis and protein digestion, ELISA, and protein mass spectroscopy [87, 88]. These microfluidic devices could efficiently analyze proteomics at very high sensitivity [89]. However, at present there are no commercially available microfluidic devices demonstrated for detecting oocyte or embryo biomarkers based on secretome differences. There is also still a lag in clinical translation of assays of specific protein candidates that have reported correlation to embryo implantation potential. Therefore, a combination of proteomic with genomic assays in a microfluidic device may lead to the next "proteogenomic" revolution for embryo diagnostics.

20.2.5.3 Transcriptomics

Complementary to proteomics analysis, single-cell transcriptome sequencing is a high-throughput tool for quantifying gene expression heterogeneity in populations of cells [90]. Since the first single-cell transcriptome sequencing (RNA-seq) was presented by Tang, many techniques have developed to perform RNA-seq on individual cells. For convenience, the molecular approaches applied in genomics, methylomics, and transcriptomics described can be summarized into four main steps: (1) cell lysis or fractionation, (2) DNA and/or RNA purification, (3) amplification, and (4) sequencing. Recently, Aaron and colleagues adapted a previously developed strategy for single-cell RNA-seq that has shown promise for superior sensitivity and implemented the chemistry in a microfluidic platform for single-cell whole-transcriptome analysis [91]. In this approach, single cells are captured and lysed within the closed microfluidic device, where mRNAs with poly(A) tails are reverse-transcribed into cDNA. Subsequently, double-stranded cDNA is collected without potential contamination. Microfluidics provides a more precise and sensitive method compared to conventional tube-based protocols. With 0.2 M reads per cell, it's capable of reconstructing a majority of the bulk transcriptome with 10 single cells; this feature makes large-scale single-cell gene expression profiling possible. Whether this technology could find application in human ART or testing of oocyte and/or embryo developmental competencies remains to be demonstrated.

20.2.5.4 Genomics

Whole-genome amplification (WGA) for next-generation sequencing has seen wide applications in biology and medicine when characterization of the genome of a single cell or few cells is required [92]. Single-cell sequencing characterizes the genome of individual cells, which is suitable for embryology to deduce information from an embryo. Microfluidics-based systems can be used to detect copy number variations (CNVs) in DNA samples derived from embryos or other cell types.

The WGA uniformity is critical for CNV detection, whereas the WGA accuracy is essential for avoiding SNV (single-nucleotide variation) detection errors, either false positives or false negatives [93]. Fu and colleagues reported a new method, emulsion whole-genome amplification (eWGA), to use the small volume of aqueous droplets in oil to improve the WGA chemistry for uniform amplification of a single cell's genome [94]. By distributing single-cell genomic DNA fragments into a large number of droplets, a few DNA fragments in each droplet are allowed to reach saturation of DNA amplification. They compared normal diploid human cells with a monoclonal human cancer cell line having inherited CNVs. The results validated that eWGA not only offered higher coverage, but also enabled simultaneous detection of SNVs and CNVs with higher accuracy. The application and integration of a microfluidic platform for gamete/embryo culture and genomic analysis has the potential to reduce molecular and operator error, but is yet to be realized. Demonstration of proof-of-concept utility of microfluidic genomic analysis in clinical ART is wanting.

20.2.5.5 Methylomics

DNA methylation is an epigenetic modification essential for normal development and maintenance of biological functions, such as gene repression, parental imprinting, X-chromosome inactivation, and suppression of repetitive genomic elements. Chemical base modification in DNA by DNA methyltransferase, specifically methylation, has been well studied as an important mechanism of epigenetics. DNA methylation is a crucial element in the epigenetic regulation of mammalian embryonic development [95]. Therefore, the determination of DNA methylation of, for example, 5′-methylcytosine in the CpG sequence in mammals has attracted attention [96, 97]. Furthermore, microfluidic technology can be applied to DNA methylation analysis because the microfluidic platform offers the advantage of making it possible to perform thousands of DNA methylation reactions in small reaction volumes, resulting in a high-throughput analysis with high sensitivity. Recently, Ronen and colleagues reported on a universal, high-throughput, microfluidic-based fluorometric assay for studying DNA methylation in vitro, utilizing methylation-sensitive endonuclease digestion of DNA substrate probes [98]. Coupling this assay with a high-throughput microfluidic platform may open new horizons for epigenetic research and even lead to novel therapies to reverse aberrant methylation patterns responsible for aberrant development and/or diseases. The microfluidic approach can provide an advantageous approach to determining DNA methylation, and epigenomic information, because of its rapid and efficient processes.

20.2.6 Microfluidics for Cryopreservation

20.2.6.1 Slow-Rate Freezing/Thawing and Vitrification/Warming

Since the first live birth from human embryo cryopreservation three decades ago [99], cryopreservation has become an important component of ARTs. Cryopreservation techniques have evolved, leading to higher success rates and the introduction of oocyte, sperm, zygotes, and embryo cryopreservation into IVF clinics worldwide. Cryopreservation of oocytes, zygotes, and embryos has significantly expanded the scope of infertility treatment. There are two basic techniques applied to the cryopreservation of human oocytes: slow-rate freezing and ultra-rapid cooling by vitrification. These techniques have been compared and discussed extensively as indicated in the following reviews [99–101].

Independent of the technique used for cryopreservation, minimizing cell damage throughout the cryopreservation process is critical to enhancing the overall outcome. Osmotic shock sustained during the loading and unloading of cryoprotectant agents (CPAs) is a major source of cell damage during the cryopreservation process. A microfluidic approach may allow greater precision in CPA exposure and reduced osmotic shock to cells during cryopreservation. Cryoprotectant agents are necessary to eliminate formation of damaging intracellular ice crystals, yet CPA exchange procedures exert osmotic stress. Osmotic stress refers to the stress experienced by cells due to changes in osmotic pressure within and outside the cell. While lethal osmotic stress during vitrification and warming has largely been overcome (high cryosurvival rates), sublethal effects remain and affect oocyte function, embryo development, and treatment outcomes. Osmotic stress is especially a challenge for oocyte and zygote cryopreservation because their fluid volumes, which need to be exchanged with CPAs, are several orders of magnitude larger than other mammalian cells. In addition, the high concentration of CPAs used in vitrification can also be problematic in relation to sublethal osmotic stress [101]. Initial work using microfluidics and cryopreservation was focused on freezing of HepG2 cells and demonstrated benefit in cell survival [102]. Song and his colleagues developed a microfluidic approach to control the loading and unloading of CPAs using diffusion and laminar flow. This microfluidic approach minimized the osmotic shock during the CPA loading phase before freezing and the CPA unloading phase after thawing. The device possesses a long microfluidic channel with three inputs and one outlet, which offers a long distance to develop a complete diffusion along the channel (Fig. 20.3). By changing injection flow rates at the inlets, the diffusion pattern along the microfluidic channels could be controlled. This microfluidic approach, using diffusion phenomena, yields concave and convex CPA concentration profiles along the channel during loading and unloading steps, respectively. The novel microfluidic-based approach improved post-thaw cell survivability by up to 25% on average over conventional cryopreservation protocols. The method developed in this study provided a platform to cryopreserve cells with higher viability and functionality and minimal intertechnician variability.

Recent studies on mature human oocyte cryopreservation demonstrated that vitrification has enhanced outcomes in terms of higher survival rates, cleavage, and pregnancy rates compared to its slow-rate freezing alternative [103]. The osmotic fluid control provided by microfluidics gives an

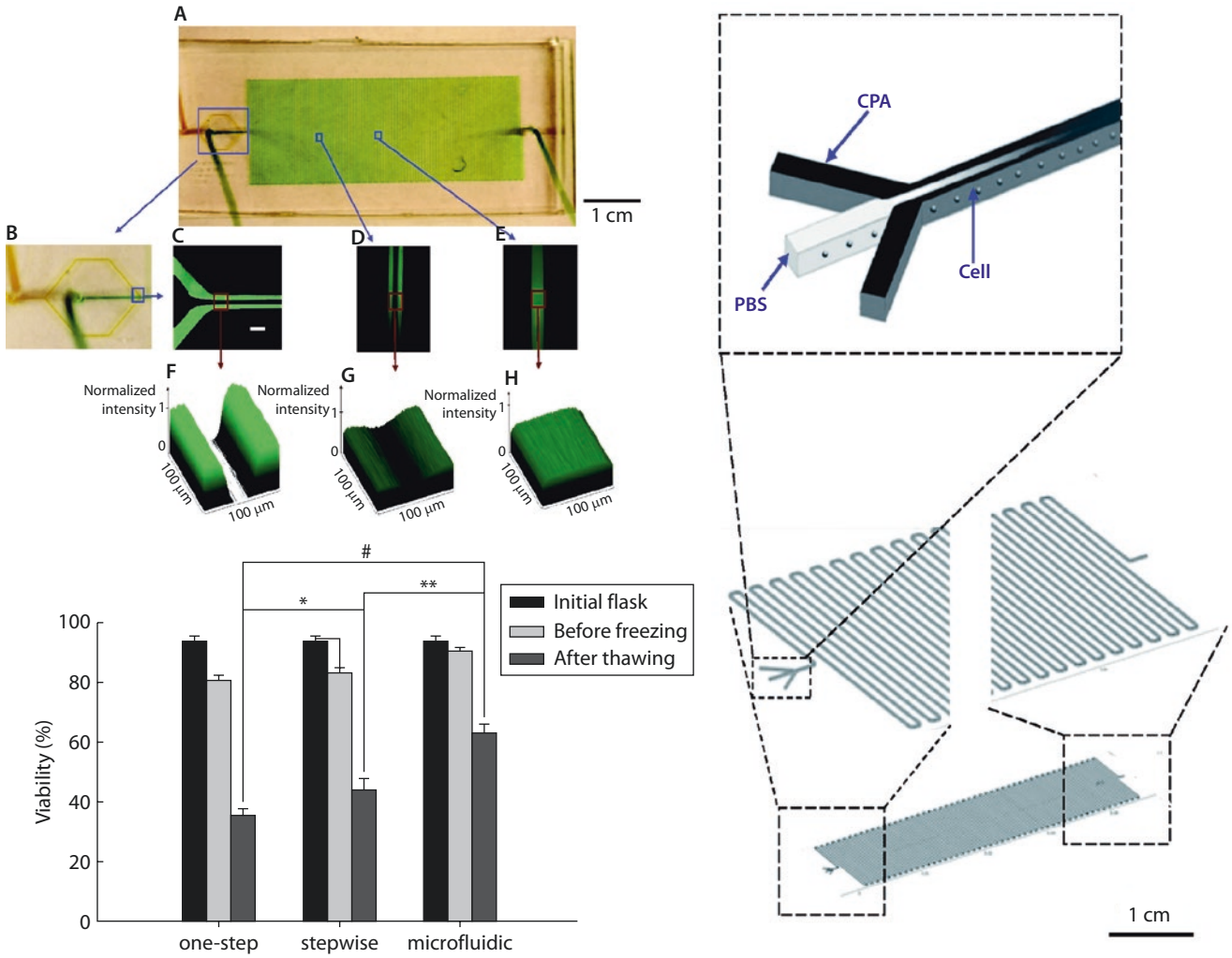

Fig. 20.3 Device structure and increased viability achieved by osmotic shock reducing during cryopreservation process [102]. Increased viability is achieved by reducing osmotic shock during cryopreservation process through gradual exposure of CPA concentration to the cells

unprecedented level of precision and osmolality control not available with manual pipetting. This is particularly important in vitrification, where higher concentrations of cryoprotectant agents (CPAs) are used. In an effort to lower the osmotic stress the cells experience, a protocol for fluid exchange was developed that moves solution over cells at increasing concentrations of CPAs to lessen the osmotic change. A minimum osmotic stress cryo-exchange profile made possible by microfluidics demonstrated that minimizing osmotic stress improved embryo development in comparison to manual pipetting vitrification protocols. Microfluidics provided the capability of continuous perfusion, essentially an infinite amount of steps, and is capable of providing real-time and continuous optical microscopy and lab-on-a-chip automation for reproducibility by eliminating operator variability. Osmotic stress was accurately predicted using computer modeling by Kedem-Katchalsky equations and known permeability parameters [104]. Recently, another microfluidic device using both permeable and impermeable solutes was designed to further confirm the remarkable accuracy of the Kedem-Katchalsky equations to model osmotic stress of oocytes and zygotes for vitrification. By using a combination of both permeable and impermeable solutes, the device was capable of controlling the shrinkage rate of the cell independent of its minimum cell volume. The study demonstrated the importance of the shrinkage rate and its effect on sublethal damages [105]. The minimization of the shrinkage rate had no effect on the cryosurvival of murine zygotes, as both manual and microfluidic CPA exposure had 100% cryosurvival. The shrinkage rate, however, did have an effect on sublethal damages. The lowering of the shrinkage rate, and therefore sublethal damage, significantly increased the developmental competence of cryopreserved murine zygotes. This study is the first to experimentally and mathematically separate the effects of the shrinkage rate from minimum shrinkage volume and the first to demonstrate reduced sublethal cell damage to zygotes with microfluidic-exchanged CPA, which resulted in improved subsequent embryo development. Studies evaluating microfluidics for thawing or warming gametes, zygotes, or embryos are still necessary to evaluate the final practical utility of microfluidics in cryopreservation.

20.2.7 Future Investigative Needs and Integration and Automation of Microfluidics

Assisted reproductive technologies have been evolving for the last several decades as a combination of assisted reproduction, cellular/molecular biology, and genomic techniques. To date, most research using microfluidics in ART has only focused on a single component of the entire IVF system, for example, sperm selection, oocyte maturation, embryo culture, fertilization, or cumulus removal. There is considerable enthusiasm about the potential applications of the microfluidic platform for assisted reproduction, as many of the important functions necessary for IVF have already been demonstrated. Indeed, only a few examples have integrated more than one step in the IVF process [65]. These results provided evidence that all phases of the IVF process could be integrated on a single microfluidic platform. A goal of microfluidic integration of all phases of IVF, in a stepwise fashion, into a singular self-contained device should be achievable. To be successful, integration must accommodate multiple concerns, such as the size difference of an expanded cumulus-oocyte complex versus a denuded zygote, the removal of cumulus cells, movement of spermatozoa in specific channel designs to optimize fertilization during coincubation with the oocyte, blastocyst expansion, and even hatching. In addition, microfluidic devices should provide medium at the optimal time, place, and flow rate to minimize gamete/embryo handling and improve outcomes. The act of microfluidic automation, in and of itself, may prove beneficial in removing potential human error and subjectivity, while at the same time providing a more biomimetic environment for improving human embryo development, pregnancy outcomes, and offspring health.

20.3 Conclusion

Application of microfluidic devices in ARTs has been shown to improve manipulation processes and developmental efficiencies. Such improvements will lead to lower costs, increased access, and decreased physical stress on gametes and embryos. Microfluidics allows oocytes and embryos to be handled in a more precise and gentler manner than traditional pipetting techniques. Instead of moving embryos from one microdrop to another, the embryo can be moved into a specified location and "parked." Then different media (maturation, fertilization, and/or embryo culture) can be moved to the embryo in either a static or dynamic flow paradigm. These media changes can be achieved gradually, reducing environmental stress on the gamete and/or embryo. Furthermore, microfluidic devices can contain much smaller volumes of media than traditional microdrop culture systems allowing the addition of expensive growth factors (if desired and warranted) at a fraction of the cost of microdrop systems. In addition to providing a more in vivo-like culture environment, microfluidic technology is ideally suited for complex embryo manipulations, such as removal of the cumulus cells, ICSI, embryo biopsy, and integration with bioassays that may evaluate genomic, transcriptomic, proteomic, and methylomic normalcy.

Although the full potential of microfluidic technology has yet to be realized for assisted reproduction, there have been many exciting developments and demonstrations of different aspects of IVF and embryo/oocyte manipulation using microfluidic principles and devices. Microfluidic techniques provide numerous advantages for biological and biomedical research, including ease of modularity, small sample requirement, potential of automation, and high throughput. One could expect that these novel single-cell techniques on microfluidic devices will be important in numerous biomedical areas. Furthermore, it allows for development of new methods for IVF and other assisted reproductive technologies. The microfluidic platform may open many potential new avenues of investigation to improve our knowledge of basic gamete and embryo physiology. Lastly, new technologies such as 3-D printing may vastly improve our ability to develop physical systems that closely mimic the in vivo environment and enable noninvasive oocyte/embryo analysis [106]. Such breakthroughs will lead to new avenues of investigation to improve our knowledge of basic embryo physiology and translate into laboratory improvement and patient care.

Review Questions

1. What are two potential benefits of using microfluidics for embryo culture?
2. Why might one use microfluidics for sperm isolation? Specifically address a functional sperm benefit.
3. What are the benefits and limitations of using microfluidics for embryo bioanalysis?
4. Can microfluidic-controlled delivery of cryoprotectants reduce osmotic stress? If so, explain the reported advantages in cryopreservation of gametes/zygotes/embryos.

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Time-Lapse Microscopy for Embryo Culture and Selection

Andrey V. Dolinko and Catherine Racowsky

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Learning Objectives

- To provide an overview of time-lapse microscopy (TLM) technology
- To introduce embryo morphology and morphokinetics that can be identified with the use of TLM
- To review the literature on the use of TLM to predict blastocyst formation, implantation, and live-birth potential
- To discuss the effects of embryo, laboratory, and maternal factors on morphokinetic parameters as observed by TLM

21.1 Introduction

The first live human birth following in vitro fertilization (IVF) was achieved with insemination of a single oocyte almost 40 years ago [1]. However, in the early years of IVF, reported pregnancy rates even among good prognosis patients were as low as 6% per retrieval [2]. In an attempt to increase pregnancy rates, controlled ovarian stimulation regimens were developed, resulting in more oocytes retrieved and embryos obtained. The trickledown effect was that multiple embryos were transferred and success rates increased, with resulting pregnancy rates being as high as 30%. However, this brought high rates of multiple-gestation pregnancies, which are associated with significant risks to fetal and maternal health. Maternal complications, including preeclampsia, gestational diabetes mellitus, and preterm labor, occur two to ten times more frequently in pregnancies with multiple fetuses than singletons. In turn, perinatal morbidity is significantly increased in multiple-gestation pregnancies, resulting in low (<2500 g) and very low (<1500 g) birth weight, and preterm birth. These result in dangerous consequences, such as cerebral palsy, retinopathy, bronchopulmonary dysplasia, polycythemia, hypoglycemia, necrotizing enterocolitis, and death [3].

To avoid these complications, elective single-embryo transfer (eSET) has emerged as the most effective method for reducing assisted-reproductive technology (ART)-associated multiple births [4]. A variety of approaches have been taken to identify the most viable embryo(s) to be transferred in eSET cycles. Two of these approaches, the evaluation of embryo morphology and embryo morphokinetics, rely on the fact that the human embryo typically develops along a predictable timeline during preimplantation development. A recent entrant into the field of ART that allows for continuous evaluation of both embryo morphology and morphokinetics is time-lapse microscopy (TLM).

21.2 TLM Technology

The basic concept of TLM is the use of a digital camera to capture images of embryos at set and frequent time intervals; these images can then be played back as a time-lapse sequence to observe embryo development and to evaluate embryo morphology and the timing of various events throughout that development. The major tenant of TLM is, therefore, that a more complete picture of embryo development can be acquired compared with conventional morphology (Fig. 21.1).

A number of TLM systems have been created, including the EmbryoScope® (Vitrolife), the Primo Vision™ system (Vitrolife), Early Embryo Viability Assessment (Eeva™, Auxogyn, Inc.), the Geri™ (Genea Biomedx), and Miri TL™ (Esco). These systems differ in important ways (Table 21.1). While the EmbryoScope, Geri, and Miri TL are stand-alone combined TLM-incubator systems, both the Primo Vision and Eeva™ are TLM systems that are installed into existing incubators. Furthermore, the systems use two different lighting techniques to photograph the embryos, bright field versus dark field.

Dark-field technology creates contrast by taking advantage of light scattering and can thus only detect cytokinesis. In contrast, bright-field technology takes advantage of the embryo absorbing light to create contrast and can therefore capture both cytokinesis and the presence of nuclei and other morphological subtleties [5]. A major advantage of this is that it allows for distinguishing large fragments and blastomeres within the developing embryo (see ▶ Sect. 21.3.2.2), as well as clearly identifying other abnormalities such as the presence of multinucleation (see ▶ Sect. 21.3.2.1).

Notably, both technologies expose embryos to less light than conventional IVF techniques and most systems eliminate short wavelengths of light below 550 nm that are known to be damaging to embryo development [6, 7]. Specifically, Eeva uses a dark-field microscope with a light-emitting diode (LED) at a wavelength of 625 nm. Eeva captures images at a single focal plane every 5 min, exposing embryos to 0.032 s of light per image for a total of 114–363 mJ/cm^2 over 3 days; this is equivalent to 15–48 s on a conventional IVF micro-

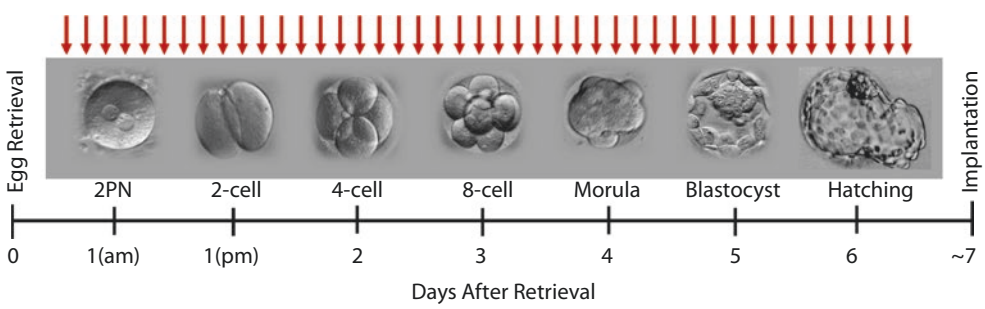

Fig. 21.1 Progression of human embryos through preimplantation development. Compared with static observations (shown by the photographic images), time-lapse microscopy allows images to be acquired at preset time intervals (red arrows; every 2–60 min), with integration into time-lapse videos and subsequent analysis

Table 21.1 Characteristics of commercially available time-lapse microscopy (TLM) systems

TLM system	Incubator type	Light source	Focal planes	Image frequency	Dish	Embryos per dish	Dishes per system	Culture type	Software
EmbryoScope®	Standalone	Bright field 635 nm	1–9	2–10 min	EmbryoSlide	12	6	Single	EmbryoViewer ± KIDScore
EmbryoScope +®	Standalone	Bright field 635 nm	11	10 min	EmbryoSlide+	16	15	Group	EmbryoViewer ± KIDScore
Eeva™	Modular	Dark field 625 nm	1	5 min	Eeva Dish	12	1	Group	Eeva Test
Geri	Standalone	Bright field long λ	1–11	5 min	Geri Culture Dish			Group	
Miri TL™	Standalone	Bright field 635 nm	3–7	5 min	Individual or Communal Reservoir CultureCoin	14 14	6 6	Single Group	Miri TL Software + Assist
Primo Vision EVO	Modular	Bright field 550 nm	3–11	5–60 min	Primo Vision Microwell Group Culture Dishes	9–16	1	Group	Primo Vision Software
Primo Vision EVO+	Modular	Bright field 590 nm	3–11	10–60 min	Primo Vision Microwell Group Culture Dishes	9–16	1	Group	Primo Vision Software

scope [8]. In contrast, the EmbryoScope uses a bright-field system with a single LED at 635 nm. This system is also more customizable, allowing embryologists to capture images from 1 to 9 focal planes with the recommended default set at acquisition of images in 7 focal planes every 10 min. At this setting, an EmbryoScope exposes embryos to 53–99 J/m^2 of light over 5-day culture, as compared to 394–776 J/m^2 in a conventional IVF microscope. The Miri TL, in turn, captures images over 0.064 s per image in 3–7 focal planes every 5 min at a wavelength of 635 nm. Finally, the Primo Vision EVO uses a green LED at 550 nm to capture images of all embryos at 3–11 focal planes every 5–60 min, while the Primo Vision EVO+ uses an amber LED at 590 nm to capture images of individual embryos at 3–11 focal planes every 10–60 min.

The systems also use different culture dishes. The EmbryoScope uses a proprietary culture dish, the EmbryoSlide. One of these dishes contains 12 individual microwells under a confluent oil cover. The EmbryoSlide+, for use with the EmbryoScope+, in turn can hold up to 16 embryos under a confluent oil cover in 2 culture compartments of 8 individual microwells under a common media droplet. The Eeva uses the Eeva Dish, which is a standard-sized petri dish with individual microwells in the center of the dish, but also sharing common medium. The Primo Vision Microwell Group Culture Dishes contains 9–16 microwells under a single drop of culture medium. Finally, the Miri TL requires either the Individual CultureCoin, which can hold up to 14 embryos in individual microwells for isolated culture, or the Communal Reservoir CultureCoin, which can hold 14 embryos in individual microwells with a culture medium and oil overlay that is shared by all embryos for group culture.

Other differences between the various TLM systems include their temperature regulation, management of oxygen and carbon dioxide tensions, and filtration systems. Each system also comes equipped with its own proprietary software for image analysis and annotation.

Regardless of the system, all can be used to evaluate embryo morphology and morphokinetics throughout the early stages of embryo development.

21.3 Embryo Morphology

To date, morphologic assessment of embryos has been the standard of care for the evaluation of embryonic developmental potential. Conventional morphologic analysis encompasses evaluation at static time points, as first described by Edwards and colleagues [9]. This allowed for the determination of developmental rate (i.e., assessing if

certain milestones are reached within a particular time frame) and morphological characteristics at particular times after insemination. Various scoring systems have been proposed for evaluating zygotes at the pronuclear stage, cleavage stage, and blastocyst stage [10]. Within these approaches, evaluation can be done either at a single time point or at several stages during development. While existing studies suggest that one or more morphological parameters can independently predict viable embryo selection, implantation rates of embryos selected and transferred on day 3 are typically no higher than about 40% [11]. This suggests that embryo morphology identified at a single or even at multiple time points is not always reflective of embryo viability [11].

In addition to evaluating embryos at the conventional time points, TLM allows for the identification of developmental milestones normally missed by traditional approaches without disturbing the system. However, a limitation of TLM systems is that they do not allow for rotation of the embryos, limiting potential morphological evaluation at any individual time point, in particular when blastomeres overlap or a high degree of fragmentation is present [12].

21.3.1 Pronuclear Stage Embryo

The first morphologic event assessed approximately 16–18 h after insemination is the presence of two pronuclei (PNs) at the fertilization check. The use of a single static time point can miss the appearance and disassembly of one or both PNs. With continuous recording, TLM can help identify the transient PNs and avoid discarding diploid zygotes that have undergone early PN disappearance or fusion of the 2PNs to form only 1PN [13]. TLM also allows monitoring of PN position within the cytoplasm. In normal development, the PNs migrate into apposition in the center of the cytoplasm, and failure to do so is associated with lower blastocyst development potential [14].

21.3.2 Cleavage Stage Embryo

Normally developing embryos undergo the first cleavage division late on day 1 and have reached the four-cell and eight-cell stages, respectively, on days 2 and 3 after insemination. Morphological assessment of cleavage stage embryos includes evaluating the number of blastomeres, the extent of fragmentation, blastomeric symmetry, multinucleation, and the presence of compaction [10]. However, these factors are fluid and can change rapidly during dynamic embryonic development, meaning the presence or absence of certain parameters is highly dependent on the timing of assessment. This opens up the possibility for using TLM to observe and track the appearance and disappearance of fragments and multiple nuclei [13].

21.3.2.1 Multinucleation

Blastomere multinucleation has been suggested as a possible marker of aneuploidy and implantation potential. Because this event is a transient occurrence, TLM can identify it with greater sensitivity than single time-point assessment. As previously mentioned, though, only bright-field TLM has the ability to visualize nuclei, while dark-field TLM is limited in this regard.

In one study, of embryos found to have multinucleation using TLM, only 27.6% (44 of 159 embryos) could be detected within the timeframes proposed by ESHRE/ALPHA consensus (22–24 h, 25–27 h, or 44–45 h postintracytoplasmic sperm injection [ICSI]) [15, 16]. Two other studies further demonstrated that multinucleation is a transient occurrence. In one study, while 43.2% of embryos demonstrated multinucleation at the two-cell stage, only 15.0% were found to be multinucleated at the four-cell stage. Of particular interest, 52% of multinucleated two-cell embryos cleaved into entirely mononucleated four-cell embryos [17]. In the other study, 42.53% of embryos were multinucleated at the two-cell stage, and only 14.47% demonstrated multinucleation at the four-cell stage, with 73.4% of the former cleaving into mononucleated four-cell embryos [18]. Of note, not all embryos that demonstrated multinucleation at the four-cell stage were multinucleated at the two-cell stage.

Multinucleation is reflective of the timing of morphokinetic parameters of developing embryos. In the studies mentioned above, embryos with multinucleation at the two-cell stage had significantly longer times from insemination to two- through five-cell stages than embryos that did not demonstrate multinucleation throughout early development ($p \leq 0.001$ in both studies).

The association between aneuploidy and multinucleation is less clear. Balakier et al. [17] found no significant difference in multinucleation between euploid and aneuploid embryos. Furthermore, nuclear correction (i.e., embryos demonstrated multinucleation at the two-cell stage and not at the four-cell stage) was seen in both euploid (78%) and aneuploid (57%) embryos, suggesting that this phenomenon is not an indicator of ploidy status [17].

The association between multinucleation and embryo viability also remains to be clarified. While Ergin and colleagues found that embryos with evidence of multinucleation at the two-cell stage had significantly lower implantation rates than nonmultinucleated embryos (23.3% vs. 43.6%, respectively, $p < 0.001$) [15], Aguilar and colleagues found no difference in implantation rate for embryos multinucleated at the two-cell stage ($p = 0.07$). However, the latter group did find a significantly lower implantation rate in embryos with multinucleation at the four-cell stage ($p = 0.001$) [18].

21.3.2.2 Fragmentation

Known causes of fragmentation include abnormal pH, temperature, and oxygen tension. With conventional assessment, as fragmentation increases, the incidence of blastocyst formation decreases. This is thought to be due to loss of

cytoplasm and associated mitochondria and cytoplasmic proteins [19]. Large fragments can be distinguished from blastomeres only by the absence of the nucleus. Unfortunately, single time point assessment can miss the presence of the nucleus, which is disassembled during the mitotic phase, thus erroneously categorizing blastomeres as large fragments. This, in turn, significantly affects the accuracy of embryo scoring [20].

While fragmentation is thought to be a marker of poor developmental potential, a recent TLM study demonstrated no difference in blastocyst or good quality blastocyst formation rates between embryos exhibiting <10% or 10–50% fragmentation when either at the two-cell or four-cell stages. This suggested that the occurrence of fragmentation during cell division is not a useful index of high developmental ability [19].

In contrast, another study investigated whether the presence of large fragments (>45 μm without a nucleus) and abnormal cell divisions (production of more or less than two blastomeres per cell division) influenced correlation between live-birth rate and number of blastomeres detected on day 2 by conventional scoring. By conventional scoring, embryos with four cells on day 2 had higher live-birth rates than embryos with less than or greater than four cells. Via time-lapse-assisted reassessment, abnormal cell divisions and/or a recalculated fragmentation of >25% was recognized in 18.3% of transferred embryos. None of these embryos resulted in live birth. Once they were excluded from the analysis, the number of blastomeres on day 2 had no impact on live-birth rate [20]. This suggests that fragmentation and abnormal cell division are crucially important to developmental potential.

21.3.2.3 Irregular Cleavage

Several abnormal cleavage behaviors have been described in the literature, including direct cleavage (<5 h from two to three cells), abnormal cleavage (origination of more than two cells from a single cell division event), chaotic cleavage (disordered cleavage behavior up to the four-cell stage), reverse cleavage (fusion of two daughter blastomeres after complete separation or failed cytokinesis after karyokinesis), disordered division (division of one blastomere in previous cleavage was delayed after the other blastomere had undergone the next cleavage), and failure to achieve six intercellular contact points (ICCPs) at the eight-cell stage. One study that evaluated irregular cleavage patterns found that 15.6% of embryos demonstrated abnormal cleavage at either the first cleavage (from one to more than two daughter cells) and/or the second cleavage (from two to five or more blastomeres, instead of four) [21]. In another study, 337 (97.7%) of 345 embryos derived from immature oocytes that underwent in vitro maturation (IVM) demonstrated abnormal cleavage patterns, including one or more of the following: direct cleavage, uneven blastomeres, fragmentation, big fragments, distorted cytoplasmic events, developmental arrest, or disordered division [22]. Importantly, a third study subsequently found low rates of good quality blastocysts (as defined by Gardner criteria) among embryos with severe abnormal cleavage patterns, including large fragmentation, developmental arrest, direct cleavage, fragmented blastomeres, asymmetric cleavage, cell-cell fusion (i.e., reverse cleavage), and mixed patterns of irregular cleavage [23].

The question arises as to whether these irregular cleavage patterns influence blastocyst formation and transfer outcomes. The first study addressing this question was performed by Athayde Wirka and colleagues, albeit with small numbers of embryos. In that study, implantation rates were lower for all abnormal phenotypes assessed (Fig. 21.2) [24]. In a subsequent study with larger numbers, Yang and colleagues found that embryos that demonstrated severely abnormal cleavage patterns, including developmental arrest, direct cleavage, disordered division, and fragmentation, had very low rates of blastocyst formation. Embryos with less severe cleavage abnormalities—such as distorted cytoplasmic movement, uneven blastomere formation, and big fragmentation formation—also demonstrated lower blastocyst-formation rates, although not as profound. A hierarchical model from A to F, depending on the timing of

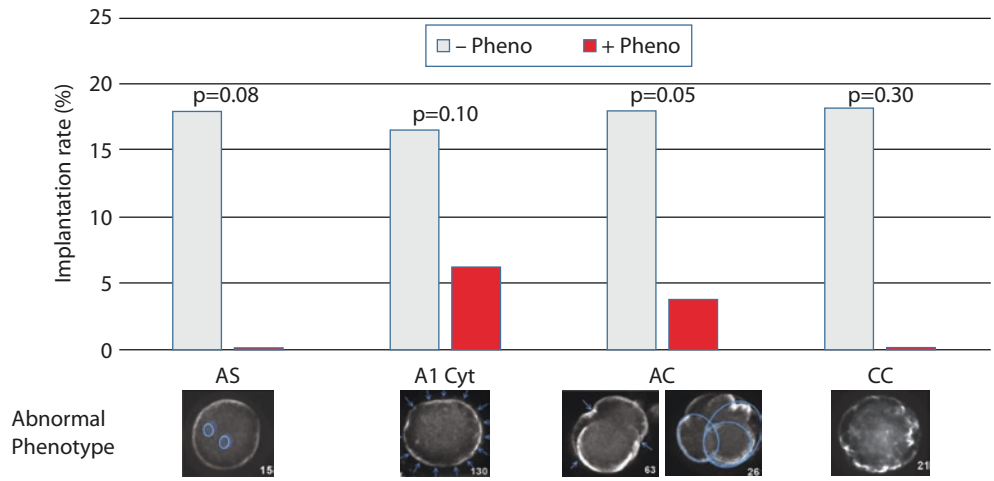

Fig. 21.2 Relationship between implantation rate and abnormal phenotypes in cultured human embryos: AS abnormal syngamy, A1Cyt abnormal first cytokinesis, AC abnormal cleavage, CC chaotic cleavage. (Adapted with permission; from Athayde Wirka et al., *Fertil Steril* 101(6):1637–1648; 2014)

an abnormal cleavage pattern (at first, second, or third divisions), was shown to have good predictive value for overall blastocyst formation (from 94.8% to 21.2%, $p < 0.001$) and good quality blastocyst formation (70.8% to 3.8%, $p < 0.001$). In a prospective observational study to validate this model, the authors demonstrated an implantation rate ranging from 67.0% down to 0.0% from Grades A to D ($p < 0.001$) [22].

In another study, Liu and colleagues found that embryos failing to achieve six ICCPs at the eight-cell stage resulted in a significantly lower proportion of eight-cell embryos at 68 h postinsemination, worse morphology scores, and reduced implantation rates [25].

Despite the above observations, a significant caveat is that the presence of irregular cleavage patterns does not preclude successful outcomes. In one study, embryos with irregular cleavage patterns were transferred in 16 women who did not have embryos with regular cleavage patterns available. These resulted in three live births [21].

21.4 Embryo Morphokinetics

TLM and continuous monitoring of embryos allow for identification of the precise timing of various milestones in embryonic development. Reported milestones include second polar body extrusion; the appearance of the 2PN and the subsequent fading of the 2PN/syngamy; cleavage to two, three, four, five, six, seven, eight, and nine cells; morula formation; start and completion of compaction; start of blastocyst cavitation/blastulation/blastocoele formation; full blastocyst formation; expanded blastocyst formation; and blastocoel cavity contractions.

Using these time points, researchers have defined a variety of calculated parameters that describe how long an embryo spends in any particular phase of development and how long it takes an embryo to go from one phase to another. Unfortunately, the nomenclature used by various authors to define these parameters is not consistent, nor are the reference time points the same from which they base their timing calculations. These inconsistencies make it difficult to compare studies, draw sound conclusions, and validate described models in new settings. To reign in these variations, two groups have proposed uniform, but distinct, sets of nomenclatures for currently identified TLM markers and potential future markers.

Notably, both nomenclatures depend on three general principles. First, they each define a standard referent for all cycles. Kaser and Racowsky [26] suggest setting the referent time point at the moment of appearance of the first cytokinesis furrow. This event is clearly identifiable regardless of whether bright-field or dark-field microscopy is used, and it is independent of the method of insemination (IVF or ICSI, see *Insemination Technique*). Ciray et al. [27], on the other hand, suggest that the referent time point should be set at the time of insemination. The disadvantage of the latter is that the mean time of injection of oocytes is typically used, which introduces inaccuracy particularly when a large number of oocytes are injected.

Second, they provide guidelines for defining all the other stages. While Kaser and Racowsky suggest that stages be defined as the "time of first definitive identification of an event," Ciray and colleagues set the time record for all events as the first or last TLM-generated frame/image at which an event is identified.

Finally, both groups suggest annotations for the derivation of time intervals and the duration of any specific stage of embryo development. Using different nomenclature, both groups suggest a general formula in which the duration of an event or phase is equal to the timing of a later developmental event minus the timing of an earlier developmental stage.

Although a few years have passed since both nomenclatures were proposed, neither has been universally adopted. Without this standardization, it has become progressively more difficult to compare studies.

21.4.1 Blastocyst Development

While blastocyst-stage fresh transfers may have higher pregnancy and live-birth rates than cleavage-stage fresh transfers [28], the cumulative live-birth rates may not be different [29, 30]. Moreover, extended in vitro embryo culture does not come without risks. In humans, ART has been associated with genome-wide disturbances of methylation reprogramming and parent-specific imprinting, including higher rates of Beckwith-Wiedemann and Angelman syndromes [31]. Extended culture and blastocyst transfers have also been shown to be associated with higher rates of monozygotic [32, 33] and monochorionic twinning [34], as well as a higher risk of congenital malformations and preterm birth among singletons [35, 36]. Thus, having the ability to predict which embryos will successfully develop into blastocysts and thus have a higher chance of implanting and leading to a live birth without exposing said embryos to extended culture is an attractive option.

The first study to suggest that TLM-monitored morphokinetic parameters can accurately predict successful blastocyst formation was published in 2010. In this study, 100 supernumerary thawed embryos were cultured in a TLM system; 33–53% developed into blastocysts over four experiments. The authors then identified that by setting certain time limits on the duration of three morphokinetic parameters—the duration of first cytokinesis, the duration of the two-cell stage, and the time interval between the appearance of cleavage furrows of the second and third mitoses (yielding a four-cell embryo)—they could accurately predict which embryos went on to reach the blastocyst stage with 94% sensitivity and 93% specificity. Unfortunately, none of these embryos were subsequently transferred for implantation. Thus, this first study did not allow any conclusions to be drawn regarding the potential for live birth of those embryos meeting these criteria compared with those whose morphokinetics fell outside these ranges [37].

Since 2010, multiple other groups have published studies that support the possibility of predicting blastocyst develop-

ment using morphokinetic parameters through the cleavage stage. Most of the early studies investigating these parameters simply evaluated the differences in timing between embryos that developed into blastocysts and those that did not. Some of those studies also evaluated the difference in timing of embryos forming high- versus low-quality blastocysts as defined by morphologic criteria.

Hashimoto and colleagues found that embryos developing into high-quality blastocysts took significantly less time to develop from the two-cell stage to the seven-cell and eight-cell stages, and accordingly spent significantly less time in the second cleavage (three- to four-cell) and third cleavage (five- to eight-cell) stages than poor-quality embryos [38]. Similarly, Dal Canto and colleagues found that embryos that successfully developed into blastocysts took significantly less time to reach seven or eight cells and spent less time in the interval between four and eight cells and in the third cleavage stage. Furthermore, of the embryos that reached the blastocyst stage, those that went on to become expanded reached the eight-cell stage significantly faster than blastocysts that did not expand [39]. Desai and colleagues also noted in a sample of 648 embryos, that those forming high-quality blastocysts reached the 8- and 9+ cell stages significantly faster than embryos that formed poor-quality blastocysts or embryos that arrested prior to blastulation [40].

Whether a difference exists regarding blastocyst formation for timing of the earlier cell divisions is more controversial. Two of the studies listed above found no significant differences in the timing of progression to the two-, three-, four-, five-, or six-cell stages for those embryos that were arrested after the eight-cell stage versus those that successfully developed into blastocysts [39] or for those that became high-scoring versus those that were low-scoring blastocysts [38]. In contrast, Cruz and colleagues showed that good-morphology blastocysts progressed through the early cleavage cycles faster than poor-morphology blastocysts, reaching the four- and five-cell stages faster. This study also demonstrated that embryos forming blastocysts progressed through these early cleavage cycles faster than embryos that arrest early, taking significantly less time to reach the two-cell, three-cell, and four-cell stages [41]. Similarly, the embryos that formed high-quality embryos in Desai and colleague's study took significantly less time to reach the two- and four-cell stages than embryos that formed low-quality embryos [40]. Accordingly, the times spent at the two- and three-cell stages were also significantly shorter in both studies.

One relatively consistent finding is that embryos with high developmental potential seem to develop faster than embryos with poor outcomes. Furthermore, faster development to the blastocyst stage appears to be on a continuum, with high-quality blastocysts developing faster than low-quality blastocysts, which in turn develop faster than embryos that arrest prior to blastulation.

Subsequent studies took the next step to determine if morphokinetic parameters can be used to predict blastocyst formation. Kirkegaard and colleagues used previously identified parameters to create models to describe embryonic potential to develop into high-quality blastocysts versus low-quality blastocysts/arrested development. Based on 571 2PNs in their cohort, the authors found that the duration of first cytokinesis, duration of the three-cell stage, and the absence of direct cleavage can predict development of high-quality blastocysts (area under the curve (AUC) of 0.63, 0.63, and 0.58, respectively). The combination of all three variables modestly improved on that prediction to an AUC of 0.69 [42]. Another study analyzed 17 morphokinetic parameters in a cohort of 3354 embryos. They found that 16 of the 17 parameters were significantly different between embryos of top and good quality versus embryos of bad quality (as defined by Gardner's classification) and arrested embryos. The authors subsequently built receiver operating curves (ROC) for each of those variables, noting that the three highest AUCs were from derived time variables, specifically the duration of time from five to eight cells, the cleavage synchronicity from four to eight cells, and the cleavage synchronicity from two to eight cells (AUC 0.778, 0.776, and 0.786, respectively) [43].

One of the seminal studies in the field of time-lapse imaging was published by Conaghan and colleagues in 2013. This study developed what is now known as the *Eeva Test*. First, in the development phase, the authors recorded embryos using the Eeva microscope and identified the timing of the first cytokinesis (P1) and the durations that the embryos are in the two-cell stage (*P*2; *t*3–*t*2, where *t* = time) and the three-cell stage (*P*3; *t*4–*t*3). They then built a classification tree model to determine optimal timing windows for embryo development to predict blastocyst formation, from which was created an automated image software that allowed the Eeva system to automatically track the embryos and provide a score based on the classification tree. This *Eeva Test* originally categorized embryos into two groups—Eeva High and Eeva Low, depending on the probability of blastocyst formation—based on two of the three parameters: P2 and P3. More recently, the test was expanded to define Eeva Medium embryos [Fig. 21.3; [44]].

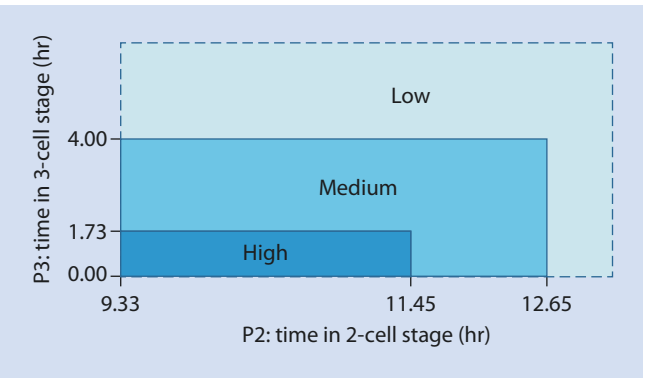

Fig. 21.3 Diagram depicting the modified *Eeva™ Test* which utilizes time in the two-cell stage (P2) and time in the three-cell stage (P3) for classification of embryos as having high, medium, or low potential to form a blastocyst. This test was designed to be used in conjunction with conventional morphological evaluation. (Drawn from data in VerMileya et al., *Reprod Biomed Online* 29(6):729–736; 2014)

Subsequently, embryologists at five different fertility centers performed further investigations of the *Eeva Test* for prediction of blastocyst formation on day 3, using either conventional morphologic assessment alone or the combination of morphology and *Eeva Test* results. In this test phase, the authors found that the adjunct information from the *Eeva Test* significantly increased both the specificity (84.7% vs. 52.1%, $p < 0.0001$) and the positive predictive value (54.7% vs. 34.5%, $p < 0.0001$) of predicting usable blastocysts over morphologic evaluation alone [8].

Since that time, multiple studies have attempted to validate these results. In the first of these validation studies, five embryologists independently predicted blastocyst formation in two cohorts of embryos using day 3 morphology alone or using day 3 morphology in combination with *Eeva Test* results. In this population, blastocyst formation rates were significantly higher in the Eeva High group than in the Eeva Low group. Having the adjunct information from the *Eeva Test* increased the odds ratio of correctly predicting blastocyst formation from 1.68 to 2.57 among embryos graded as good or fair based on day 3 morphology alone [45]. Taken together, these data support the utility of the *Eeva Test* as an adjunct to day 3 morphology in choosing embryos able to form blastocysts.

Subsequently, Aparicio-Ruiz et al. [46] retrospectively analyzed a cohort of 3002 embryos derived from oocyte donation cycles. In this study, the authors evaluated blastocyst formation based on morphology up to day 3 as defined by the ASEBIR (asociación para el Estudio de la Biología de la Reproducción) criteria and/or a modified *Eeva Test* classification. The Eeva classification in this study stratifies embryos into three groups as suggested by VerMilyea and colleagues [44]. Using Eeva classification alone, the AUC to evaluate blastocyst prediction was 0.717. In turn, logistic regression of blastocyst potential based on the ASEBIR morphology categories alone yielded an AUC of 0.728, and using the Eeva classification as an adjunct to ASEBIR only modestly increased the AUC to 0.788 [46]. Thus, available data indicate that the *Eeva Test* only modestly improves prediction of an embryo to blastulate, although it may serve as an adjunctive tool.

While the *Eeva Test* was the first to be created and marketed as an adjunctive blastocyst prediction tool, other groups have also attempted to develop their own blastocyst predicting models. Milewski and colleagues observed 432 embryos from 77 patients as they developed in an EmbryoScope and noted the timing of the following parameters: the time to two through five blastomeres and the timing intervals from two to three blastomeres and three to four blastomeres. The values for each of those six parameters were divided into quartiles and assigned a score from 0 to 2. In this cohort, univariate logistic regression demonstrated that all of the parameters are significantly associated with blastocyst development. Using the most significant values, the authors carried out a multivariate analysis to create a score based on time to two and five blastomeres and the time an embryo spends in the two-cell stage. This combined score was then divided into quartiles, and significant differences were observed among quartiles in the percentage of embryos that developed to the blastocyst stage, with values ranging from 18% to 90% ($p < 0.001$). Of note, the combined score was significantly higher for embryos that developed to the blastocyst stage than those that did not. An ROC based on this combined score yielded an AUC of 0.806. The authors then validated this model on an independent set of 271 embryos, 116 of which formed blastocysts. The results of the ROC analysis were similar with an AUC of 0.813, and the combined score values for embryos that developed to blastocyst were significantly higher than for arrested embryos [47].

Motato and colleagues [48] in turn performed an observational retrospective study of 7483 zygotes that developed into 3215 blastocysts from 990 first treatment ICSI cycles. The authors classified the embryos according to two binary timing variables: time to morula formation (81.28–96.00 h) and t8–t5 (≤8.78 h). A hierarchical regression model based on these two variables yielded an AUC of 0.849, with the rate of blastocyst formation decreasing from 84.4% in the embryos where both times fell within the defined parameters to 13.8% in the embryos where their timing of development fell outside those ranges.

Another group also used a big-data approach to develop a blastocyst prediction algorithm. Using data collected from 24 clinics on 3275 embryos transferred on day 3, the authors created an algorithm (KIDScore) using the following parameters: the number of pronuclei equals 2 at the one-cell stage, time from insemination to pronuclei fading at the one-cell stage, and time from insemination to the two-, three-, five-, and eight-cell stages. The embryos were then ranked into five groups based on the derived algorithm to yield an AUC of 0.650, with a sevenfold difference in implantation rate between KIDScore 1 (5.18% implanted) and KIDScore 5 (36.17% implanted). The authors then validated the study on data from 11,218 embryos cultured to day 5 in 31 clinics and noted that the KIDScore algorithm could predict blastocyst formation with an AUC of 0.745 and blastocyst quality with an AUC of 0.679 [49].

In turn, Storr and colleagues created an algorithm in their own embryo population to predict the quality of blastocysts based on Gardner criteria. They found that the timing of their synchrony stage (i.e., time to eight blastomeres minus time to five blastomeres) was the single best individual predictor prior to embryo compaction of a top-quality blastocyst (i.e., inner cell mass and trophectoderm grades AA or BA), with an AUC of 0.585. As they measured timing parameters all the way through the blastocyst stage, the time to expanded blastocyst was the best predictor of top-quality blastocysts regardless of embryo stage (AUC 0.727). Accordingly, a model based on the third synchrony stage (time between division to five cells and subsequent division to eight cells), the time to eight cells, and time to expanded blastocyst could predict the formation of a top-quality blastocyst with an AUC of 0.748 [50]. This model, though, is of limited clinical utility as it is dependent on the embryos developing to the blastocyst stage in vitro.

Notably, Storr and colleagues also attempted to validate the model developed by Cruz and colleagues in 2012 (see above). Unfortunately, none of those parameters had any statistically significant predictive ability in the new embryo population. Only the duration of the second cell cycle could predict top or good quality blastocysts with an odds ratio of 1.88 (95% CI 1.11–3.19) [50].

Wang and colleagues, who found low rates of good quality embryos among those with severe abnormal cleavage patterns (see ▶ Sect. 21.3.2.3), also developed a hierarchical embryo selection model based on the development of 1728 embryos. In univariate logistic regression models, they found that the time to pronuclei disappearance, two, three, four, and five blastomeres, and the lengths of the second cell cycle (time to transition from two-cell to three-cell embryo) and third cell cycle (time to transition from three-cell to four-cell embryo) were all significantly associated with good quality blastocyst formation. Subsequently, in a multivariate logistic regression model, the time to five blastomeres (≤49.07 h) and the length of the third cell cycle (≤0.50 h) were the most predictive values. A hierarchical embryo selection model that began with excluding embryos with severe abnormal cleavage patterns and then selected embryos based on the length of the third cell cycle and the time to five cells found that embryos that were within the timing ranges were likely to develop into good quality blastocysts 68.8% of the time, as compared to only 35.71% of the time for embryos that were out of range [23].

21.4.2 Implantation Potential

While it is useful to predict blastocyst potential or blastocyst quality early in embryo culture, showing that TLM can help predict implantation would be much more indicative of the technology's potential. A limitation of the conclusions discussed below is that implantation rate is defined differently in various studies, with some studies simply defining it as simply a rise in serum beta across two consecutive blood tests or a positive urine hCG 14 days after single embryo transfer [23], while others evaluated patients for the presence of a gestational sac with or without a fetal heartbeat at various stages of gestation.

The first study to show that early embryo morphokinetics may differ between embryos that went on to implant and those that did not showed that embryos that implanted trended toward a faster appearance of the nucleus in the first blastomere after cleavage. These embryos also had a significantly faster time to synchrony (time from nuclear appearance in first blastomere to nuclear appearance in second blastomere) [51].

21.4.2.1 Meseguer's Hierarchical Model

In 2011, Meseguer and colleagues published a seminal paper evaluating the timing parameters of 247 embryos with known implantation data. The authors found significant differences in the timing of the early cleavage stages; embryos that implanted reached the two- through five-cell stages faster than embryos that did not implant, and they spent less time as two-cell and three-cell embryos. The authors then divided the exact timing of those early cleavage cycles by quartiles and established optimal ranges reflecting the highest probability of implantation (which were not necessarily found in the fastest-dividing embryos). Using these quartiles, the authors created a hierarchical model of embryo grading based on morphokinetic parameters (time to five-cell stage, duration of the two-cell stage, and duration of the three-cell stage) and morphologic exclusion criteria. The embryo categories ranged from A through E, with implantation potentials decreasing from 52% down to 8% across the categories [52].

The same group subsequently performed first a retrospective study and then a randomized controlled study (RCT) to evaluate the hierarchical model that they developed. In both studies, the group compared implantation rates of embryos cultured in an EmbryoScope and then selected for transfer using the hierarchical embryo grading system to embryos cultured in a standard incubator and selected using conventional morphologic grading. In the retrospective study, the authors noted that embryos cultured in and selected using the TLM system had significantly higher implantation rates than embryos from the conventional incubator group (adjusted odds ratio 1.201, 95% CI 1.059–1.363, $p = 0.0043$) [53]. In the RCT, the authors reached similar conclusions, with the implantation rate of transferred embryos greater in the TLM group than the conventional morphology group (RR 1.43, 95% CI 1.05–1.39, $p = 0.02$) [54].

Using the data from the RCT, the authors also prospectively validated their hierarchical embryo grading system. They showed that implantation rates had a direct relationship with the morphokinetic category, decreasing from 52.9% in Category A to 13.7% in Category E embryos [55] and have since further refined it (◘ Fig. 21.4; [56]).

Unfortunately, other groups have not been successful in reproducing the results of Meseguer's group in their own populations. Yalcinkaya and colleagues performed a retrospective analysis using Meseguer's hierarchical model on 910 transferred embryos with known implantation data cultured in an EmbryoScope. They found that the highest pregnancy rates were in groups C+ and A- (48.2%) and the lowest rate in group E (19.7%). Of note, the laboratory techniques used in this study differed from those used by Meseguer's group, including different media (single step versus sequential culture) and oxygen concentrations (~20% vs. 7.0%) [57].

Similarly, Freour and colleagues attempted external validation of Meseguer's hierarchical model in an unselected population with various transfer days (days 2, 3, 4, 5, or 6). The first noteworthy difference was that the overall implantation rate in this study was lower than in Meseguer's study (22% vs. 38%, respectively), given the fact that the latter included patients with better prognoses. Evaluating the model itself, the authors found that the correlation coefficients were significantly lower in this study than those in

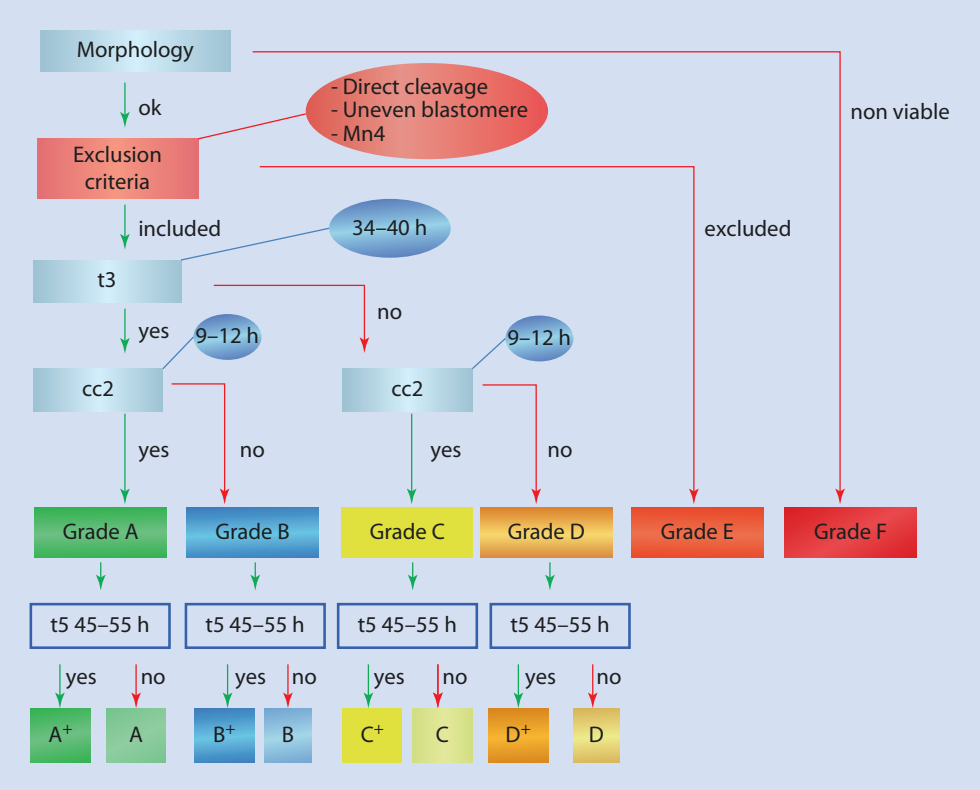

Fig. 21.4 Hierarchical classification of embryos based on conventional morphological screening, time-lapse observed morphologic criteria, and three morphokinetic parameters. Expected implantation potential increases based on classification of an embryo from right to left. (Published with permission; from Basile et al., *Hum Reprod* 30(2):276–283; 2015)

the original publication ($r = -0.66$ overall, -0.52 blastocyst stage transfer, and -0.54 cleavage stage transfer vs. -0.92; $p < 0.001$) [58].

In a small RCT of 64 patients, Kahraman and colleagues randomized good prognosis patients to an EmbryoScope group (embryos cultured in an EmbryoScope and selected for transfer based on morphology and morphokinetics using Meseguer's model) or a conventional incubator (with selection based on day 5 morphology alone). The authors found no difference in blastocyst development rate, the rate of good and top quality blastocysts, or ongoing pregnancy or miscarriage rates between the two groups. Furthermore, there was no difference in the morphokinetic parameters between implanted and nonimplanted embryos [59].

Collectively, the above results suggest that each lab may need to create its own TLM algorithm based on its own techniques and patient population.

21.4.2.2 Eeva Test Extension/Validation

Several studies have attempted to use the *Eeva Test*, originally developed for the prediction of blastocyst development (see ► Sect. 21.4.1), to predict implantation potential. One group performed a retrospective analysis of the *Eeva Test* at three different clinics with various lab parameters (i.e., the Eeva scores were blinded and not considered during embryo selection). While the implantation rates were different at each clinic, collectively, Eeva High and Eeva Medium embryos had significantly higher implantation rates than Eeva Low embryos (37% and 35% vs. 15%; $p < 0.0001$ and $p = 0.0004$, respectively) [44].

In contrast, another group did not find such positive results with the *Eeva Test*. Using an EmbryoScope, rather than the Eeva microscope, Kirkegaard and colleagues analyzed data from 1518 transferred embryos with known implantation outcomes at seven clinics from three countries. They retrospectively grouped embryos into usable and nonusable based on the timing parameters for Eeva High and Eeva Low embryos, respectively. The odds ratio for implantation between usable and nonusable was 1.60, with an AUC of 0.57 for prediction of implantation. Notably, 50.6% of embryos that implanted would have been categorized as nonusable by the model. As the authors write, "this hypothetical experiment illustrates the risks of defining too narrow time intervals for optimal division in order to achieve a high specificity at the expense of a low sensitivity" [60].

More recently, Adamson and colleagues reported the results of a nonrandomized prospective concurrent-controlled pilot study in which they compared the combination of automated TLM assessment using the *Eeva Test* and day 3 morphology (TLM was also used to exclude abnormal cleavage behaviors) to day 3 morphology alone. In this study, the test group had a significantly higher implantation rate than the control group (30.2% vs. 19.0%, $p = 0.003$). Patients in the test group who had at least one Eeva High embryo had significantly higher implantation rates than patients with only Eeva Low embryos (36.8% vs. 20.6%, $p = 0.03$). Furthermore, among morphologically good embryos, Eeva High embryos were significantly more likely to implant than Eeva Low embryos (44.1% vs. 20.6%) [61]. A notable limitation of this study is that different culture dishes were used in the two groups.

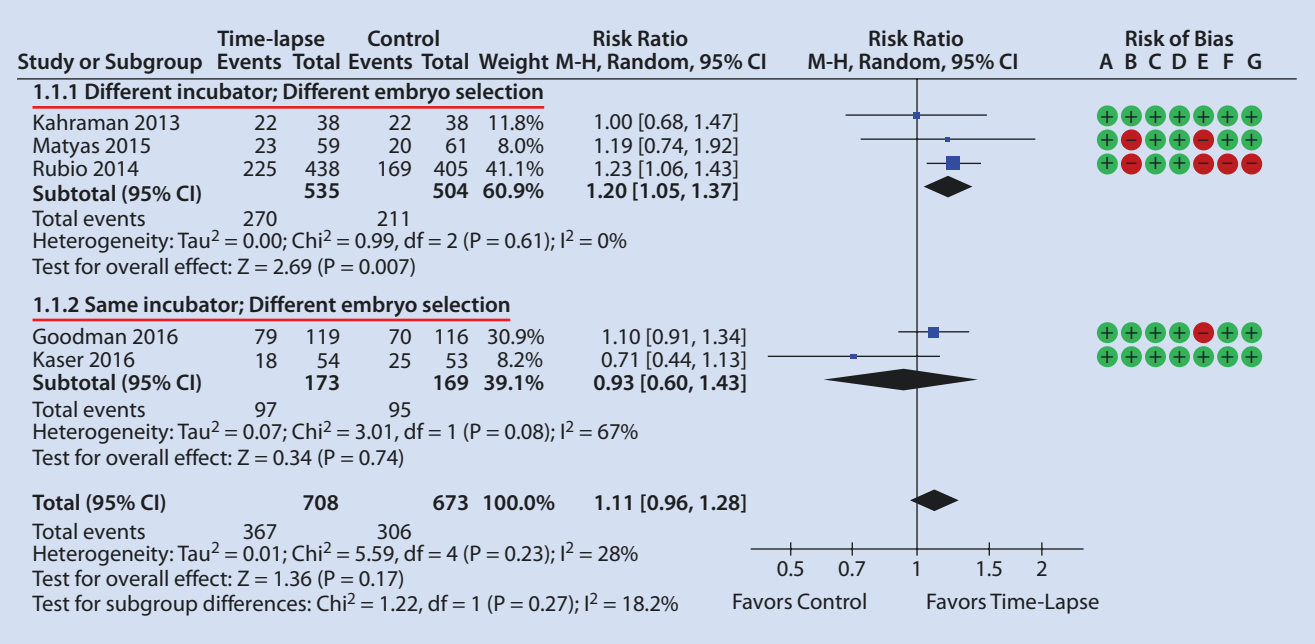

Fig. 21.5 Forest plot of ongoing pregnancy per woman randomized in randomized controlled trials to date. Risk of bias legend: (*A*) selection bias, (*B*) performance bias, (*C*) detection bias, (*D*) attrition bias, (*E*) reporting bias, (*F*) other bias. (Updated from Racowsky et al., *J Assist Reprod Genet* 32(7):1025–1030;2015)

Aparicio-Ruiz and colleagues, who performed a successful validation of the *Eeva Test* for blastocyst formation (see ▶ Sect. 21.4.1), subsequently transferred embryos based on the *Eeva Test* and standard morphology evaluation. The study found increasing implantation rates across Eeva categories after both day 3 (Low 26.1%, Medium 31.7%, and High 38.2%) and day 5 transfer (Low 31%, Medium 50%, and High 66.7%). Using the combination of *Eeva Test* and day 3 morphology allowed the authors to predict implantation with an AUC of 0.650 [46].

In a large prospective, observational, two-center pilot study with a propensity matched control group, Kieslinger and colleagues also evaluated the *Eeva Test*. In the test group, embryos underwent continuous culture with selection based on *Eeva Test* and morphology. In the control group, embryos were exposed to interrupted culture with selection based on morphology alone. The authors found no difference in implantation rate, clinical pregnancy rate, ongoing pregnancy rate, or live-birth rate between the groups. However, they did note that for patients who received suboptimal quality embryos (≠8 cells or ≥ 25% fragmentation), Eeva testing led to higher implantation, clinical pregnancy, and ongoing pregnancy rates. Furthermore, the ongoing pregnancy rate was significantly higher in the Eeva High (35.3%) and Eeva Medium (41.8%) embryos than in Eeva Low (18.4%) [62]. These results suggest that although Eeva testing may not be universally applicable, it may play a role in embryo selection for transfer in patients who have no optimal embryos.

Of note, all the above studies were either retrospective or prospective and observational. To date and to our knowledge, only one RCT has been performed to evaluate the *Eeva Test*. The results of this recent pilot RCT do not support the utility of this test as an adjunct in embryo selection [63]. In this trial, adjunctive use of the *Eeva Test* with conventional morphology on day 3 failed to result in clinical outcomes equivalent to day 5 selection with conventional morphology alone. When applied on day 5, the *Eeva Test* yielded lower pregnancy rates than selection with conventional morphology alone on day 5. Moreover, a systematic review and meta-analysis of published TLM studies failed to show an improvement in implantation rate when TLM is incorporated into an embryo selection algorithm (relative risk (RR) and 95% confidence intervals (CI): RR 1.20; 95% CI 1.05–1.37 [64]; and such clinical equipoise remains after updating this meta-analysis with the recent, yet-to-be-published Eeva RCT (RR, 1.11; 95% CI = 0.96, 1.28; Fig. 21.5).

Based on the above discussion, we conclude that further well-designed RCTs are required to determine the value of incorporating TLM with conventional morphology for embryo selection.

21.4.2.3 Other Implantation Potential Studies

Other groups have also explored the potential application of TLM for prediction of implantation potential. In a retrospective analysis of 270 embryos with known implantation after day 3 transfer, one group proposed a hierarchical deselection model based on poor conventional morphology, abnormal cleavage patterns as identified by TLM (direct cleavage, reverse cleavage, or <6 intercellular contact points at end of four-cell stage), <8 cell at 68 h postinsemination, duration of the three-cell stage, and time to five cells. The proposed model had an AUC of 0.762 for embryos graded A+ to F, with no significant difference between IVF and ICSI embryos. The

authors then prospectively validated the model on 66 embryos with known implantation data using two different culture media, yielding AUCs of 0.750 and 0.820 for each media and an overall AUC of 0.783 ($p < 0.001$) [65].

Milewski and colleagues, building on their prior algorithm (see ► Sect. 21.4.1), created a predictive equation using time to two and five blastomeres and the length of the second cell cycle to yield a combined score. Stratifying the combined score into quartiles, the authors found significant differences in pregnancy rates, ranging from 16.9% to 36.0% ($p = 0.009$). The combined score values were significantly different between embryos that implanted and those that did not, although the AUC of 0.61 was not as high as the AUC of 0.806 for blastocyst prediction. Furthermore, the differences in distribution of morphokinetic parameters between embryos that implanted and those that did not were not clearly apparent [66].

Recently, Goodman and colleagues published one of the few existing RCTs in this field. The authors randomized embryos to either conventional once-daily morphology embryo evaluation or additional TLM evaluation for selection. All embryos were cultured in an EmbryoScope. For the control group, embryologists had the ability to scroll through the seven focal planes at 42, 66, 90, 114, and 148 h postinsemination, but they were restricted from viewing time-lapse footage. In the TLM group, embryos that had suitable morphology were also graded based on morphokinetic parameters, with negative points assigned for a second cell cycle shorter than 5 hrs, multinucleation, and irregular division and positive points for falling within prespecified time limits to reach the five-cell stage and the start of blastulation, the time spent as a three-cell embryo and a five- to eight-cell embryo within certain time limits. The study noted a tendency toward increased clinical pregnancy and implantation rates in the experimental group, although the differences were not significant [67].

21.4.3 Live-Birth Potential

The ultimate goal of IVF, though, is for patients to take home a healthy live-born infant. Unfortunately, few of the TLM studies published to date have explored this outcome.

In one retrospective analysis of patients allocated to embryo culture in an EmbryoScope as part of an RCT, Ahlstrom and colleagues assessed conventional morphology using EmbryoScope images and retrospectively annotated morphokinetic parameters [68]. In univariate analysis of morphology, each additional four-cell embryo available and presence of early cleavage both significantly increased odds of a live birth. Conversely, increases in grade of fragmentation at 43–45 h postinsemination significantly decreased the odds of a live birth. Univariate analysis of morphokinetics found that multiple parameters were significant predictors of live birth with shorter times in all, although these yielded poor AUC values.

In a linear regression model, the authors found an AUC for live-birth prediction of 0.73 based on early cleavage and fragmentation grade (i.e., morphological annotations). The AUC based on time to two blastomeres alone was 0.67. Combining all three yielded an AUC of 0.73, which was no different from morphological evaluation alone.

In a classification tree model using the time spent as a one-cell embryo, the time to pronuclear fading, and the time spent with a pronucleus, the authors increased the AUC to 0.83. However, on validation, the AUC dropped to 0.61. Taken together, these data suggest that morphokinetic evaluation does not outperform morphological evaluation, and may actually do worse.

Notably, these authors also attempted to apply the *Eeva Test* to live-birth prediction. They found no significant differences between groups, and in fact showed a tendency to a lower live-birth potential in the Eeva High group (17.2%) than in the Eeva Low (23%) or Eeva Medium groups (24.1%) [68].

In turn, Siristatidis and colleagues performed a prospective cohort study of TLM monitoring in PrimoVision versus conventional monitoring. In the study group, embryos were selected only by TLM monitoring rather than in combination with morphological evaluation. The authors found significantly higher clinical pregnancy (65.7% vs. 39%), ongoing pregnancy (55.7% vs. 31.3%), and live-birth rate (45.7% vs. 28.4%) in the TLM monitoring group than in the conventional morphology group. However, live birth in this study was defined as a viable pregnancy at 20+ weeks gestation, rather than as a live-born infant, thereby limiting its interpretation [69].

21.5 Embryo Factors

21.5.1 Aneuploidy

Aneuploidy is a common occurrence in human embryos, with estimates ranging from 20% to 90% depending on the woman's age [70]. The only currently existing method to identify aneuploid embryos prior to transfer involves invasive embryo biopsy with genetic screening. Any possibility of identifying these embryos using a noninvasive technology such as TLM and subsequently excluding them from transfer would be most beneficial. Unfortunately, existing data regarding the ability of TLM and morphokinetic analysis to discriminate between euploid and aneuploid embryos are conflicting.

Some studies show no significant differences in morphokinetic parameters between euploid and aneuploid embryos. One early study showed no difference in time to syngamy, duration of first cytokinesis, first cleavage, duration of two-cell stage, timing of eight-cell stage, or the start of cavitation between five euploid and four aneuploid embryos [71]. Several large studies—each evaluating between 152 and 454 embryos—similarly found no significant differences in multiple morphokinetic parameters [72–75].

However, some published studies do support the utility of morphokinetic analysis for aneuploidy screening. In 2012, Friedman and colleagues first reported that embryos with abnormal morphokinetic parameters are more likely to be aneuploid [76]. In a follow-up publication, the same group analyzed the ploidy status of each blastomere at the four-cell stage among 53 embryos after tracking three morphokinetic parameters—duration of the first cytokinesis, time from two to three blastomeres, and time from three to four blastomeres. The authors noted that aneuploid embryos with meiotic errors exhibited a greater spread of times in all three morphokinetic parameters and that aneuploid embryos with mitotic errors had greater variation in timing of two of the three parameters than euploid embryos [77].

Several other studies also found significant differences in the timing of division of euploid and aneuploid embryos. Unfortunately, these studies do not agree on whether euploid embryos divide faster or slower than aneuploid embryos. Two groups found that multiple morphokinetic parameters are significantly shorter in euploid embryos [78, 79]. Another group found the exact opposite, with aneuploid embryos dividing faster than euploid embryos [80]. To add to this mix, a fourth study found that some timing parameters were shorter in euploid embryos while others were longer [81].

Despite these uncertainties, two groups have attempted to create models based on morphokinetics to predict embryo ploidy status. Noting significant differences in the time to start of blastulation and formation of full blastocyst, Campbell and colleagues proposed a classification model for unscreened embryos, with the risk of aneuploidy increasing from low (probability 0.37) to medium (probability 0.69) to high (probability 0.97) [78]. In a follow-up study, the same group retrospectively applied this model to embryos from 69 infertile couples. Embryos in the low-risk group had significantly higher rates of implantation (72.7% vs. 25.5%, $p < 0.0001$) and live birth (61.1% vs. 19.2%, $p = 0.01$) than embryos in the medium-risk group. None of the embryos that were retrospectively classified as high-risk implanted [82]. This was the first study to show that TLM-derived morphokinetics could potentially be used to risk-stratify embryos for aneuploidy and correlate this information with a clinically significant outcome. However, a separate group of researchers was not able to successfully validate Campbell's algorithm. In a retrospective analysis of a cohort of 106 blastocysts with known ploidy status, Kramer and colleagues found that observed aneuploid frequencies were significantly different from those predicted by the model for all three risk categories ($p < 0.02$), and they were not significantly different from uniform expectation in each group. Furthermore, this group noted that interpatient variability in the timing of most morphokinetic markers was greater than intrapatient (interembryo) variability [83].

Another group also attempted to devise a euploid-embryo predicting algorithm. Evaluating the morphokinetics of 504 embryos (71.7% aneuploid rate) from 125 patients undergoing preimplantation genetic testing for aneuploidy (PGT-A), Basile and colleagues found significant differences in the time it took euploid and aneuploid embryos to reach the five-cell stage, the amount of time embryos spent in the two-cell and three-cell stages, and the time to go from the two- to the five-cell stage. After defining optimal ranges for these three-derived parameters, the authors created a hierarchical model which subdivided embryos into four categories (A–D). In this model, the likelihood of normal chromosomal content decreased serially from 35.90% (Category A) to 9.80% (Category D) [80]. Given that only 1/3 of embryos demonstrating the three optimal morphokinetic milestones were euploid, this specific model is unlikely to supplant PGT-A. Furthermore, two other research groups failed to find significant differences between the four categories defined by this model after attempting to perform retrospective validation in their own clinics [73, 74].

21.5.2 Embryo Gender

The ability to noninvasively identify embryo gender could be beneficial to selecting appropriate embryos for transfer in situations involving sex-linked disorders. Published literature, however, has not supported this possibility. Two studies have found no significant differences in the morphokinetics of male and female embryos [84, 85]. Nevertheless, one of the studies attempted to use logistic regression to identify embryos more likely to be female. Based on the time it takes an embryo to go from three cells to four cells and the time to morula formation, Bronet and colleagues proposed four categories, ranging from 71% to 42% in the likelihood of an embryo being female. Unfortunately, the two middle categories (B and C) both resulted in 50% chance of female gender, suggesting that the model cannot differentiate between genders [84]. No other studies have yet attempted to validate this particular model.

More encouragingly, Bodri and colleagues found that female gender was associated with late cleavage (time to eight cells), time to morula, and time to several blastocyst stage variables. After defining optimal timing parameters for the latter, the authors could predict 74–78% of female embryos [86]. Although encouraging, these results remain to be validated.

21.6 Laboratory Factors

As is well known, developing embryos are particularly sensitive to environmental factors. Laboratory techniques are thus designed to maintain maximum stability of optimum in vitro conditions. The effect of various techniques on embryo development is thus important to study, not only to define their effect on the ultimate outcome of embryo development (i.e., blastocyst formation, implantation, live birth, and long-term health) but also on how embryos develop under particular conditions.

21.6.1 Culture Media

Several studies have explored the effect of culture media on embryo morphokinetics. As TLM allows for undisturbed embryo culture, three studies have explored the effect of single-step vs. sequential media. One of these studies with randomization of sibling embryos to single versus sequential media showed significantly faster progression to time of pronuclear fading and time to the two- through five-cell stages in single media [27]. Another study found conflicting results, with embryos grown in a single-step medium taking longer to develop to seven and eight cells and from three to four and five to seven cells. Furthermore, another group reported no difference in most of the measured morphokinetic parameters [87]. In support of these results, a third group found no difference in any measured morphokinetic parameters for embryos grown in a single medium with or without renewal on day 3 [88].

Furthermore, none of the three studies found significant differences in blastocyst formation rate, in the proportion of good quality blastocysts, or in implantation, pregnancy, or live-birth rates between single step and sequential media [27, 87, 88].

Based on these data, the morphokinetics of early embryo development does not appear to be significantly impacted either by use of single versus sequential media or by whether single medium is renewed or not.

21.6.2 Insemination Technique

Early studies suggested that ICSI-derived embryos progress through the early stages of development faster than IVF-fertilized oocytes. However, it was not until the advent of continuous TLM monitoring that this could be studied in depth.

As with other studies on morphokinetics, the results of existing studies are conflicting. Early on, Hashimoto and colleagues showed no difference in the time required for second cleavage (three to four cells) and third cleavage (five to eight cells) between conventional IVF insemination and ICSI [38]. Another group showed that although embryos generated by IVF took longer to reach the two- and three-cell stages than ICSI-derived embryos, the former spent less time as two-cell embryos; thus, cleavage kinetics were realigned from the four-cell stage up to the eight-cell stage [39].

In a larger study, Cruz and colleagues cultured oocytes inseminated by IVF (n = 622) or ICSI (n = 581) in a TLM system, either immediately after insemination (ICSI) or starting on day 1 after confirmation of fertilization (IVF). When the time of reference was set at the time of insemination, ICSI-derived embryos appeared to reach pronuclear fading and the two-, five-, seven-, and nine-cell stages significantly faster than IVF-derived embryos. However, when the reference time was reset to a clearly identifiable standard, i.e., time of pronuclear fading, all differences in embryo kinetics between groups disappeared [89].

These results are in concordance with the findings of Bodri and colleagues. Before setting a clearly defined reference time, the authors found that ICSI-derived embryos develop faster during the early cleavage stages (up to four cells). However, when the reference time was once again set to time of pronuclear fading, differences in cleavage-stage parameters disappeared. However, this was not so for progression to the blastocyst stage; even after standardization, the timing of each blastocyst-stage parameter was significantly shorter among IVF-fertilized embryos than after ICSI [90].

Thus, more research is still required to determine whether different optimal timing parameters need refinement based on method of fertilization. However, these studies do reinforce the fact that the nomenclature used for TLM studies needs to set a clearly identifiable stage in development as the referent time for all morphokinetic parameters (reviewed by [26]).

21.6.3 Blastomere Biopsy

Blastomere biopsy is an invasive method used to isolate embryonic genetic material for analysis of aneuploidy and specific genetic disorders. It has been shown that removing two cells versus one cell from cleavage-stage embryos results in a lower blastocyst formation rate, suggesting that blastomere biopsy is detrimental to embryo development [91, 92]. Several morphokinetic studies have reinforced these results.

In the first study, embryos spent significantly more time in the stage at which they were biopsied than did the unbiopsied control embryos. Subsequently, biopsied embryos took significantly longer to reach later stages in development, including compaction, morula formation, early blastocyst formation, and full blastocyst formation [93]. A second study confirmed these results, finding that blastomere biopsy significantly delayed the timing to the first cleavage after biopsy, as well as the time to compaction and the start of blastulation. This study also noted that blastomere biopsy also affected postembryo transfer development, with embryos that did not implant demonstrating a significant delay in both the time of morula formation and the start of blastulation as compared to embryos that implanted successfully [94].

21.6.4 Cryopreserved Oocytes

As elective fertility preservation has emerged as a viable option for many women, an increasing number of embryos in the coming years will be derived from thawed cryopreserved oocytes. One study noted that embryos derived from fresh oocytes reached the two- to four-cell stages faster than embryos derived from thawed vitrified oocytes, although no differences were observed in the time to five cells or in the duration of the two- and three-cell stages [95]. In another study, embryos derived from thawed versus fresh oocytes showed no significant difference in the timing of nine developmental events or five measured time intervals up to the five-cell stage [96].

21.6.5 Stimulation Cycle Medications and Hormones

The maternal hormonal milieu may also significantly affect oocyte quality and embryo development. Several studies have explored this issue by concentrating on embryo morphokinetics.

The first study noted no difference in any morphokinetic parameters between embryos obtained from stimulation cycles using follicle stimulating hormone (FSH), human menopausal gonadotropin (HMG), or both. However, as the dose of recombinant FSH (rFSH) increased, the longer it took for embryos to reach the two-cell, the 6 through 9+ cell stages, to begin blastulation, and to complete maximal blastocyst expansion. Furthermore, differences in serum estradiol concentration were significantly associated with the timing of most developmental milestones through the time of blastulation. Serum progesterone levels, in contrast, were associated only with differences through the first four cell division cycles [97].

The same group subsequently explored whether the medications used for controlled ovarian stimulation and ovulation trigger affected early embryonic developmental kinetics. While embryos derived from GnRH agonist with hCG trigger cycles took longer to reach the two- through five-cell stages compared to embryos derived from a GnRH antagonist with GnRH agonist trigger cycle, differences in timing did not persist further [98].

In a follow-up to this study, a separate group specifically explored whether the ovulation trigger used affects embryo kinetics. The authors found that embryos reached several developmental milestones significantly faster after a GnRH agonist trigger versus an hCG trigger, while other milestones and developmental phases were not affected [99].

Based on these studies, we can conclude that the clinical manipulation of maternal hormones is associated with embryo kinetics. However, more studies are needed to tease out these affects and their impact on efficacy of TLM algorithms for embryo selection and clinical outcomes.

21.7 Maternal Factors

21.7.1 Age

Increasing maternal age is generally associated with decreasing oocyte quality, increased risk of aneuploidy, and resultant decreasing embryo quality. Based on limited data, though, maternal age does not seem to have an effect on embryo developmental kinetics. One study showed no significant differences based on donor age for times required for embryos to go from three to four cells and from five to eight cells [38]. A separate study found no difference in any morphokinetic parameters between embryos that successfully implanted from women <35, 35–37, or ≥ 38 years old [100].

21.7.2 Body Mass Index (BMI)

Obesity has been shown to be correlated with lower implantation and pregnancy rates following ART [101, 102]. The effect of obesity on embryo kinetics is currently inconclusive.

Specifically, Bellver and colleagues found no significant kinetic differences between embryos derived from obese (BMI ≥ 30 kg/m^2) versus normal weight (BMI = 20–24.9 kg/m^2) women with infertility. After subsequently categorizing the embryos in each group based on Meseguer's hierarchical model (see ► Sect. 21.4.1), they also found no significant differences between groups in the proportion of embryos in each category (A–E) [101].

In contrast, Leary and colleagues noted that while embryos from overweight women (BMI ≥ 25 kg/m^2) are less likely to complete development postfertilization than embryos from normal weight women (BMI < 25 kg/m^2), those that do develop tend to reach the morula stage significantly faster. Interestingly, those embryos developed into significantly smaller blastocysts with fewer cells in the trophectoderm and displayed an altered metabolic pattern [103].

21.7.3 Polycystic Ovary Syndrome (PCOS)

Similarly to obesity, PCOS has significant effects on gene expression and defects in oocyte maturation [104]. The effect of these changes on embryo kinetics has been explored in a couple of studies to date.

Wissing and colleagues used TLM to evaluate embryo morphokinetics among three groups of women: 20 normo-ovulatory controls, 25 women with hyperandrogenic PCOS, and 26 women with normoandrogenic PCOS. The authors noted that embryos from hyperandrogenic PCOS seemed to develop slower through the early cleavage stages, although no differences were found between groups by the morula stage of development [104].

On the other hand, Sundvall and colleagues found that embryos from women with PCOS took significantly less time to initiate compaction and reach the morula stage than embryos from normo-ovulatory women. They noted no significant differences in the other timing parameters after adjusting for age, BMI, fertilization method, and male infertility. Interestingly, though, the proportion of multinucleated cells at the two-cell stage was significantly higher among PCOS embryos than non-PCOS embryos [105].

Given the conflicting findings of these two studies, further research is essential to discern if PCOS has detrimental effects on embryo kinetics.

21.8 Conclusions

Time-lapse imaging of embryos offers the opportunity to evaluate embryo morphology and kinetics with a noninvasive method in an undisturbed culture environment. Much insight

into early developmental characteristics has been revealed with this technology. Indeed, over 20 unique morphokinetic variables for normal embryo development have been described to date, and TLM has helped identify numerous abnormal morphological events that may otherwise be missed by static morphologic evaluation. Some of these markers have been found to be predictive of blastocyst development or implantation, while others have been identified as atypical markers of embryos with lower developmental potential. Nevertheless, despite the development of several TLM algorithms designed to predict implantation potential, unfortunately, few of the incorporated parameters have been successfully validated in multiple studies and none of the algorithms have been found to be consistently predictive of live birth, the ultimate goal of ART.

Based on existing studies, it is unlikely that TLM will supplant the existing standards of care of conventional morphologic evaluation with or without PGT-A. However, TLM may eventually evolve as an effective adjunct laboratory technique to enhance the deselection of otherwise morphologically normal-appearing embryos and the selection of the most developmentally competent embryo that is most likely to result in pregnancy and birth. Prior to that occurring, though, existing models need to be validated further, and additional well-designed prospective and randomized controlled trials need to be performed to determine whether or not use of TLM results in improved implantation and live-birth rates. Without such validation, we take the position that TLM should continue to be considered experimental and that patients should not be charged to have their embryos time-lapse imaged.

Review Questions

1. What are the different types of time-lapse microscopy (TLM) systems and what are the advantages and disadvantages of each?
2. What morphological characteristics have been described and/or better defined with the help of TLM?
3. What are the characteristics of an effective nomenclature system for TLM?
4. What parameters have been found to be predictive of blastocyst development? What parameters make up the *Eeva Test* and how has the *Eeva Test* been validated?
5. What types of models have been described for prediction of implantation potential? Please describe hierarchical selection models.
6. What are the conclusions of studies that have evaluated the use of TLM to predict live birth?
7. Can TLM be used to predict aneuploidy? How about gender?
8. Does insemination technique affect morphokinetic parameters? How about blastomere biopsy?
9. Do any maternal factors affect embryonic morphokinetic parameters?

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Sperm Assessment, Processing and Selection

Contents

Sperm Assessment: Traditional Approaches and Their Indicative Value

Tania R. Dias, Chak-Lam Cho, and Ashok Agarwal

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Learning Objectives

- Traditional approaches of semen assessment represent the cornerstone in the initial evaluation of male fertility potential.
- Macroscopic evaluation of semen includes assessment of liquefaction, age, appearance, color, viscosity, volume, and pH.
- Microscopic evaluation includes sperm agglutination, motility, concentration, morphology, and presence of non-sperm cells.
- Adjunct tests including assessment of vitality, presence of white blood cells by peroxidase staining (also called Endtz test), and fructose tests may provide additional information in specific clinical scenarios.
- Current status of computer-assisted sperm analysis (CASA).
- Overview of recently developed home testing and smartphone-based kits for semen analysis.

22.1 Introduction

Male subfertility and infertility affect many couples during their reproductive age. The prevalence of these conditions has been increasing over the years [1], representing a major burden for health care systems [2]. This has been related to intentional delayed child-bearing by couples, particularly in developed countries, as well as environmental and lifestyle factors [3]. When a couple seeks medical counselling and assistance to achieve conception, workup of female factors usually comes first since it is commonly perceived that female factors are more significant in predicting the choice and success of assisted reproductive technology (ART) [4]. However, the recognition of male factors has been increasing and they contribute either directly or indirectly to 20–70% of infertile couples [4].

Semen analysis is the key investigation of infertile men because it reflects the overall functionality of sperm production by the testes, the patency of the genital tract, as well as the secretory activity of all accessory glands [5]. This information contributes to the effective establishment of individual diagnoses [6] and serves as the essential basic investigation of male factors in an infertile couple [7].

Standardization of semen analysis constitutes an important aspect of the investigation, not only to obtain valid and useful information but also to minimize intra- and inter-laboratory variations. The World Health Organization (WHO) defined reference values for several characteristics of human semen that are widely followed since the publication of the first manual in 1980. These reference values have been revised over the years, with the most recent and fifth update being issued in 2010 [8]. The latest WHO recommendations are based on semen parameters from approximately 2000 fertile men, from eight countries and three continents, whose partners had achieved pregnancy within 12 months of unprotected sexual intercourse [9]. This represents the first attempt to set reference values in an evidence-based approach. However, the one-sided criteria used to define normality in this edition, as shown in Table 22.1, led to criticism and controversy among clinicians and researchers, due to the huge switch in the definition of the lower cut-off limits for normality relative to previous editions [10, 11]. The inclusion of only semen analysis results from fertile patients in formulating the reference values is another pitfall. The WHO criteria should not be regarded as standard but rather a recommended reference for interpretation of human semen analysis results. It is likely that the reference values need to be revised regularly based on more recent data in addition to technical advances in semen analysis. Therefore, the reference limits for semen analysis should not be over-interpreted to distinguish fertile men from infertile men [12]. A specific diagnosis should be analyzed within the context of a patient's clinical history and physical examination.

Attempts have been made to improve "manual" semen assessment by laboratory technicians by incorporating more complex and innovative technological equipment and software to reduce individual subjectivity. However, despite all the advances, computer-assisted sperm analysis (CASA) techniques are still not widely used due to lack of accuracy and efficiency [13]. In this chapter, the conventional techniques used in routine semen analysis at Andrology laboratories, taking into account the reference values defined by

Table 22.1 Reference values for human semen assessment according to the WHO fifth edition criteria[a]

Parameter	Reference value
Liquefaction	15–30 min
Semen age	0–60 min
Color/appearance	White-gray/opaque
Viscosity	Disperse drops (thread <2 cm long)
Volume	≥1.5 mL
pH	≥7.2
Total motility (progressive + non-progressive)	≥40%
Progressive motility	≥32%
Sperm concentration	$\geq 15 \times 10^6$/mL
Total sperm count	$\geq 39 \times 10^6$/ejaculate
Undifferentiated round cells	$< 1 \times 10^6$/mL
Morphology (normal forms)	≥4%
Vitality	≥58%
Peroxidase-positive leukocytes	$< 1 \times 10^6$/mL
Seminal fructose	≥13 µmol/ejaculate

[a]World Health Organization (WHO) guidelines for human semen analysis (low reference values, fifth centile, 95% confidence intervals) [8]

WHO 5th edition criteria, are presented. Semen analysis adjunct tests, as well as the recent advances in CASA and its clinical role, are also discussed.

22.2 Conventional Semen Analysis

Semen analysis comprises a series of steps in the following sequence: collection, macroscopic evaluation, and microscopic evaluation (Fig. 22.1) [14]. A proper collection of the specimen and subsequent liquefaction are crucial steps for semen assessment as this procedure attempts to mimic the natural deposit of semen in the female reproductive tract as much as possible. If liquefaction does not occur properly, the physical and chemical properties of the specimen may be compromised [15]. Following liquefaction, simple characteristics such as semen age, appearance, viscosity, volume, and pH constitute the macroscopic analysis. Finally, microscopic evaluation consists of assessment of agglutination, presence of undifferentiated round cells, motility, concentration, and morphology [9, 16]. Adjunct tests including evaluation of vitality, presence of leukocytes, and concentration of seminal fructose may be helpful for specific patients depending on the clinical feature [17]. Although the coexistence of multiple altered seminal parameters increases the risk for infertility *per se*, it is important to understand that semen characteristics should be interpreted in the context of the patient's clinical history [7].

22.2.1 Specimen Collection for Diagnostic Purposes

The collection of the specimen influences the quality of the ejaculate and is dependent on the method used. Collection of semen should ideally mimic the in vivo situation. In practice, the collection of fresh ejaculate into a non-toxic sterile plastic container by masturbation represents a non-physiological situation and is a compromise for convenience. Under natural conditions, the male partner delivers the ejaculate in a sequential manner into the female reproductive tract [18]. The first fraction is from the bulbourethral glands that function as a lubricant to the urethra. This is followed by the emission of spermatozoa from the epididymis and prostate secretions that are expelled through the urethra in several boluses [19]. The initial spurts represent the richest sperm fraction of the ejaculate. In the following spurts, there is a gradual dominance of fluid from the seminal vesicles, as well as a gradual decrease in the amount of spermatozoa [20]. In contrast, for diagnostic purposes, the sample is collected at the clinical laboratory or at home and all the semen secretions are mixed at a single time into a specific container [14]. Ideally, the specimen collection should be made in a diagnostic laboratory under rigorous quality control standards. However, for the patient's convenience, there are also commercially available kits for sample home collection. There is evidence supporting the production of a better quality ejaculate when it is collected at home [21].

The most important issue of sample collection is the abstinence time, i.e., the number of days without ejaculation. Before sample collection, spermatozoa are stored in the epididymis until ejaculation. However, the epididymal storage is not completely emptied in a single ejaculation and the remaining spermatozoa stay until the next ejaculation [9]. On the other hand, there is an accumulation of spermatozoa in the absence of ejaculation, which will ultimately be reabsorbed. Ideally, the duration of sexual abstinence before the collection should be 2–3 days [8]. There is evidence supporting the use of shorter abstinence periods for certain ART procedures, which could be particularly important for oligozoospermic men [22].

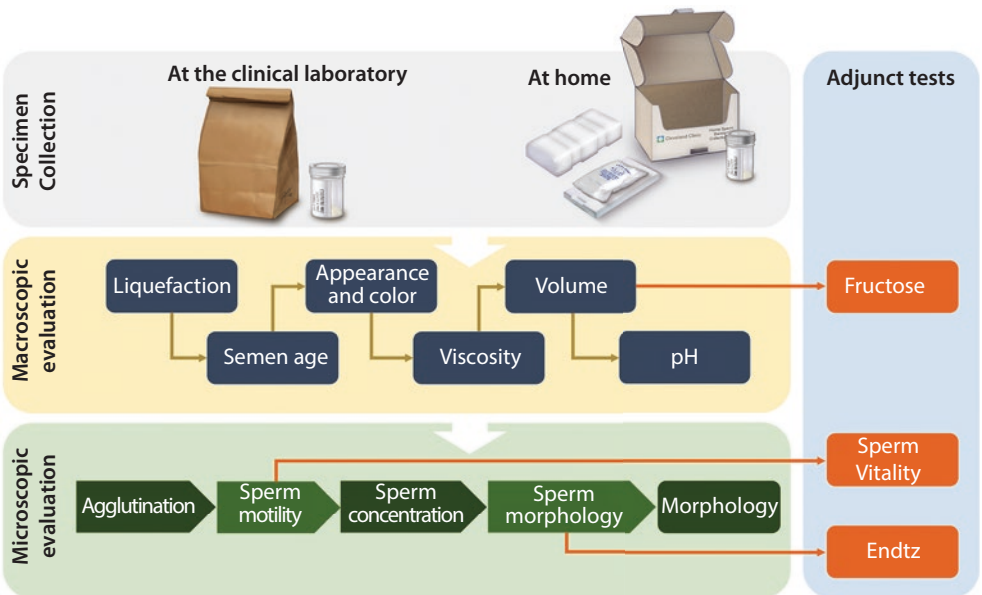

Fig. 22.1 Schematic representation of the semen analysis sequence, collection, macroscopic evaluation, and microscopic evaluation, as well as common adjunct tests performed when some parameters of semen analysis are abnormal

Due to the biological variability between samples from an individual, at least two separate seminal analysis are analyzed to achieve a definitive and accurate diagnosis [7]. The interval between separate semen analyses is arbitrary and is generally recommended to be of 2–4 weeks. Moreover, the same abstinence period is advised for repeated sample collections to facilitate comparison between results [23].

22.2.1.1 Collection at the Clinical Laboratory

Instructions for sample collection should be given to patients in advance. The specimen should be collected by masturbation into a wide-mouthed container and without using any lubricants or condoms with spermicidal agents [8]. Complete specimen collection is important since an incomplete collection may lead to misinterpretation of the results (e.g., hypospermia) [24]. Furthermore, the sample should be collected in a private room near the laboratory to minimize the transportation time to the laboratory. Time of collection should be noted by patient or laboratory staff.

22.2.1.2 Home Collection

For home collection, the same recommendations should be followed. Patients should be advised to record the time of collection and the specimen should be brought to the laboratory within 1 h [14]. Extra care should be taken during the transport of the sample by keeping it close to the body to maintain physiological temperature. The use of any condoms should be avoided. However, in the case of inability to produce a sample by masturbation and to avoid losing the first portion of the ejaculate by means of *coitus interruptus*, non-spermicidal condoms or silastic (silicone and elastic) sheaths may be used [25]. These condoms are specifically developed for specimen collection, although their toxicity to spermatozoa still needs to be scientifically tested.

22.2.1.3 Sample Handling at the Laboratory

The technician should record all the details of semen collection, including the patient's name; time of collection; time received; date, place, and method of collection; abstinence length; and completeness of collection. The clear and accurate recording of the information will avoid misinterpretations. During semen analysis, the technician should practice universal precautions to avoid contamination. The analysis should be completed within 60 min in a stepwise consecutive manner to avoid the possible deterioration of semen parameters.

22.2.2 Macroscopic Evaluation

Macroscopic semen analysis should begin with the examination of liquefaction, preferably within 30 min to 1 h after collection. Delay in processing may lead to dehydration or alterations in semen quality due to temperature and environmental changes since the specimen is being deposited in a non-natural environment. Routine macroscopic evaluation includes a quick examination of liquefaction, semen age, appearance, color, viscosity, volume, and pH.

22.2.2.1 Liquefaction

After ejaculation, human semen forms a loose gel-like coagulum in a few seconds [15] by the crosslinking of seminal vesicle proteins, semenogelins (I and II), and fibronectin [26]. The formation of this coagulum entraps spermatozoa to minimize the back-flow after deposit into the vagina and protects them from the acidic vaginal environment and immune attack. Subsequently, prostate-specific antigen (PSA) cleaves this clot to release the spermatozoa [27]. The ability of this enzyme to gradually digest the coagulum dictates the liquefaction time, which varies between a few minutes in vivo to 15–30 min in vitro [8].

Liquefaction can occur at room temperature, though it is recommended to maintain the specimen at 37 °C. Continuous gentle mixing or rotation of the sample container may facilitate the liquefaction process.

How Can It Be Evaluated?

Immediately after collection, the appearance of ejaculate is typically a gelatinous mass. During liquefaction, different components can be distinguished and the sample is described as a suspension of small gelatin-like drops in a fluid [28]. The sample is allowed to liquefy at room temperature or in the incubator at 37 °C for 15–20 min. The specimen is considered completely liquefied when homogenous and presenting a watery consistency [9].

What If Liquefaction Does Not Occur?

When liquefaction does not occur within 30 min, the sample should be incubated for an additional 30 min either at room temperature or in a 37 °C incubator [8]. An incompletely liquefied sample is suboptimal for semen analysis [17]. If a semen sample fails to liquefy within 60 min, this is defined as delayed liquefaction [9] and additional mechanical or enzymatic actions are needed to break down the coagulum and increase fluidity (see ▶ Sect. 22.2.2.4).

Clinical Interpretation of Delayed Liquefaction

Delayed liquefaction may indicate an incomplete collection of the ejaculate. Losing the last portion of the ejaculate may be a possibility. If the collection of the specimen was complete and it fails to liquefy, it may indicate impairment of the secretory activity of male accessory glands, in particular the prostate [29]. An abnormal liquefaction may also signify ejaculatory duct obstruction (EDO), congenital absence of the seminal vesicles (CASV), or genital tract infection [29, 30].

22.2.2.2 Semen Age

Semen age is given by the time interval between the collection and the start of the semen analysis after liquefaction. Semen age should not exceed 60 min to avoid deterioration of sperm quality due to environmental factors [8].

22.2.2.3 Semen Appearance and Color

A normal liquefied semen sample has a homogeneous appearance and a white-gray opaque color [8]. When it appears more transparent than usual it may indicate a low sperm concentra-

tion. Sometimes the semen sample may appear reddish-brown due to the presence of red blood cells (hematospermia) [29]. A yellowish color may occur in man with jaundice or after the ingestion of certain foods, vitamins, or drugs [9].

22.2.2.4 Viscosity

After liquefaction, the viscosity of the sample should be tested. Liquefaction and viscosity constitute distinct semen parameters with different clinical interpretations. While physical property of semen changes over time from a semi-solid to a liquid state during liquefaction, viscosity refers to the elastic properties of the sample after liquefaction.

How Can It Be Evaluated?

Viscosity of the semen can be evaluated by using a disposable plastic pipette of approximately 1.5 mm diameter. After aspirating a portion of the semen sample, it should be allowed to drop with gravity by pressing slowly. When small disperse drops are observed without the formation of any semen threads, the viscosity is considered as normal [8]. Conversely, the formation of a semen thread with more than 2 cm in length indicates hyperviscosity [15]. Hyperviscosity can be graded as slight, moderate, or high according to the length of the thread formed. Semen samples with a thread length of 2–4 cm are considered slightly hyperviscous; samples with a thread length of 4–6 cm are categorized as moderately hyperviscous; and those with a thread length greater than 6 cm are labelled as highly hyperviscous [15].

How to Proceed in Case of Hyperviscosity?

Hyperviscous semen specimens generally show elastic and sticky properties, reflected by the strong adhesion to the pipette tip while trying to aspirate, and its consistency will not change with time. This condition should be treated before further analysis as it may compromise sperm handling and quality during the assessment of sperm motility and concentration. To reduce viscosity, semen can be mechanically or enzymatically processed. Mechanical approach involves dilution of the sample with an equal volume of physiological medium followed by repeated pipetting [15]. These techniques are more effective in samples with slight and moderate hyperviscosity since vigorous and repetitive pipetting may seriously damage spermatozoa. For highly hyperviscous samples, reduction of viscosity can be achieved by the addition of proteolytic enzymes such α-chymotrypsin [31]. The enzyme α-chymotrypsin (5 mg) can be added to a hyperviscous semen sample (requirement of 0.5–5 mL in volume), which should then be incubated for an extra 10 min at 37 °C [32].

Clinical Interpretation of Abnormal Viscosity

Semen hyperviscosity is estimated to occur in 12–29% of the patients [15] and may reflect accessory glands dysfunction. Abnormal viscosity has been correlated with hypofunction of the prostate or seminal vesicles as their secretions are most abundant in seminal plasma. On the other hand, infections and inflammation of accessory glands or other male fertility-related diseases may also lead to semen hyperviscosity in men with functionally normal accessory glands [31].

Hyperviscosity impedes normal sperm motility and the tension of the trapping effect may interfere with the amount of energy needed to achieve a certain velocity, thus compromising sperm quality [15]. Although the underlying mechanisms by which hyperviscosity affects male fertility still need clarification, it has been associated with lower fertilization rates during ART, disrupted embryo development, and pregnancy failure [33].

22.2.2.5 Volume

Semen volume gives an indication of the overall functioning of the accessory sex glands [17]. Interestingly, the secretions from seminal vesicles are expected to decrease with age, thus having a major contribution to the decrease in the ejaculated volume over time [34]. According to the WHO manual, a sample is considered normal when the volume is at least 1.5 mL [8]. Calculation of total number of spermatozoa and undifferentiated round cells in an ejaculate requires an accurate measurement of semen volume. The ejaculated volume also varies with the abstinence length. The WHO recommends an abstinence period of 2–3 days prior to semen analysis [8].

How Can It Be Measured?

The volume can be measured using a graduated disposable serological pipette or by aspirating the entire specimen into a graduated tube.

Clinical Interpretation of Abnormal Volume

The semen volume is considered abnormal when it is less than 1.5 mL. A falsely low semen volume may be the result of incomplete collection and short period of abstinence. Ejaculatory duct obstruction and congenital bilateral absence of the vas deferens (CBAVD) are important differential diagnoses resulting in less fluid secretion [29]. Transrectal ultrasonography (TRUS) allows the evaluation of seminal vesicles and ejaculatory ducts and may be helpful in diagnosis [23]. Partial retrograde ejaculation represents another possibility of low semen volume. The complete lack of ejaculate is defined as aspermia [35].

22.2.2.6 pH

The measurement of pH constitutes an important part in semen analysis. While secretions from seminal vesicles are basic in nature, prostatic secretions are acidic. The predominant volume of secretion from seminal vesicles dictates the overall alkaline pH of semen under normal circumstances [17]. Although the reference value established by the WHO for semen pH is a one-sided value of ≥7.2, the pH should not be higher than 8 [8].

How Can It Be Measured?

For pH measurement, a drop of 3 μL of semen should be spread onto the pH paper (pH range 6.0–8.0), and the resultant color should be compared with the calibration strip [14].

Clinical Interpretation of Abnormal pH

Abnormal pH may indicate dysfunction of the accessory glands or incomplete collection of the ejaculate or an infection if pH exceeds 7.8 [8]. Conversely, for semen samples with pH <7.0, low volume, and low sperm numbers, ejaculatory duct obstruction and CBAVD are the differential diagnoses [36].

22.2.3 Microscopic Evaluation

The microscopic assessment gives information regarding the quality of sperm as well as the presence of non-sperm cells, including epithelial cells, immature germ cells, leukocytes, and debris. Microscopic evaluation of semen includes assessment of sperm agglutination, motility, concentration, morphology, and the presence of mucus strands and round cells (immature germ cells and leukocytes). The liquefied sample is thoroughly mixed in the original container, and only a small representative portion of the sample (about 50 μL) will be used for microscopic evaluation [37].

22.2.3.1 Agglutination

Agglutination means the attachment of motile spermatozoa to each other forming clumps. Different sites of attachment can be observed: head-to-head, head-to-tail, tail-to-tail, or mixed [8].

How Can It Be Evaluated?

A wet preparation is prepared by adding 10 μL of semen onto a clean slide and covering it with a coverslip. The dimension of the coverslip should be standardized according to the amount of sample used to create an approximate depth of 20 μm, which is ideal for sperm to move freely. The preparation should be left untouched for a few seconds, so the sample can stop drifting. Examination of the slide should be made under phase-contrast microscope at ×100 magnification [8].

Clinical Interpretation

In normal samples, no agglutination is present. The presence of any agglutination should be reported as it may interfere with the assessment of semen parameters and may indicate an immunological infertility [8]. Additional evaluation of anti-sperm antibodies is recommended in the presence of large and gross sperm clumps (see ▶ Chap. 23).

22.2.3.2 Sperm Motility

Sperm motility is one of the most important parameters when assessing sperm quality as it is an indicator of spermatozoa fertilizing competence [38]. Different types of sperm movement can be distinguished in a semen sample: progressive motility, non-progressive motility, or no motility [9]. While progressive motility describes a very active "space-gaining motion" in which spermatozoa can move linearly or in a large circle, non-progressive motility consists of all other patterns of motility [8]. These two categories of movement do not consider the speed of the spermatozoa to avoid individual bias and are mostly analyzed together as a measure of total motility.

How Can It Be Evaluated?

Sperm motility should be assessed as soon as possible after liquefaction, preferably within 30 min after ejaculation. Longer periods up to 1 h are acceptable but should be avoided to limit the bias from dehydration or changes in pH and temperature. At a clinical setting, disposable sperm counting chambers (Fig. 22.2a) are the most popular because they allow a one-step analysis for both sperm motility and concentration [14]. A drop of 5 μL of liquefied semen sample is loaded onto a counting chamber and examined under phase-contrast microscope with an eyepiece grid (Fig. 22.2b), at a magnification of ×200 in a minimum of five different fields [14]. The total number of progressive motile sperm should be counted first, followed by the total number of non-progressive motile sperm, and finally the total number of immotile sperm [8]. A laboratory counter is recommended for scoring and only intact spermatozoa should be considered. A minimum of 100 intact cells should be counted in the total of the 5 fields regardless of the category. The percentage of total motility is obtained by dividing the number of total motile cells (progressive + non-progressive) by the total number of cells counted (motile + immotile), multiplied by 100 [8].

Clinical Interpretation of Abnormal Motility

Men with low total sperm motility (<40%) and/or sperm progressive motility less than 32% are classified as asthenozoospermic. A falsely low motility may occur in a non-liquefied or hyperviscous sample. Low total or progressive motility can also be due to a delayed analysis [8].

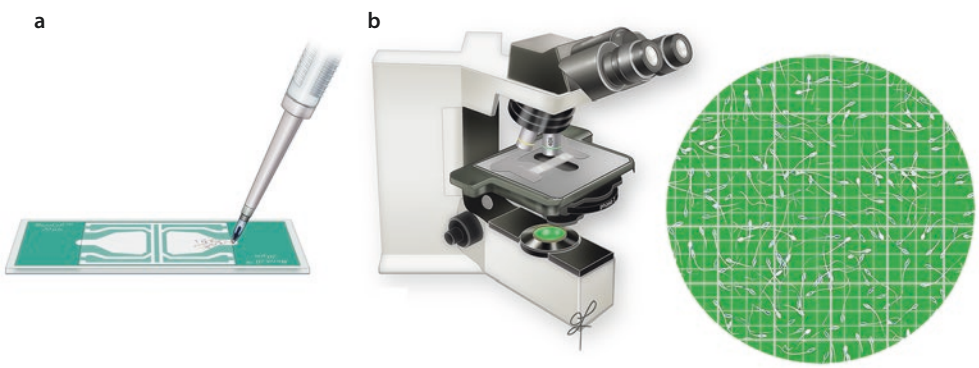

Fig. 22.2 Example of a disposable sperm counting chamber **a** and its analysis under a phase-contrast microscope with an eyepiece grid **b**

Abnormal motility may indicate ultrastructural sperm defects, including morphological deficiencies, or necrozoospermia [7]. These abnormalities can be induced by oxidative stress, which damages the sperm cell membrane and axonemal structure of sperm flagellum [39]. The presence of antisperm antibodies has also been associated with poor motility, mostly due to sperm clumping by agglutination [40, 41]. Primary ciliary dyskinesia is a less common cause of asthenozoospermia [7].

22.2.3.3 Sperm Concentration

Sperm concentration is defined by the number of spermatozoa per mL of ejaculate [8]. Sperm concentration should not be confused with total sperm count, which is defined by the total number of spermatozoa per ejaculate (sperm concentration multiplied by semen volume). In contrast to sperm concentration that is influenced by the functioning of other reproductive organs, total number of sperm per ejaculate is a direct measure of testicular sperm output. In addition, total number of progressively motile spermatozoa can be calculated by multiplying the total number of spermatozoa in the ejaculate by the percentage of progressively motile cells. The parameters of total number of sperm and progressively motile sperm in an ejaculate are widely adopted in the selection of ART treatment [42].

How Can It Be Evaluated?

Sperm concentration can be assessed using a hemocytometer (100 µm deep) [8]. However, it is quite laborious and time consuming. A large variation in proficiency testing also hinders its clinical use [43]. The one-step approach with disposable counting chambers is a reliable option that is adopted by many diagnostic laboratories. Several parameters have to be considered when using disposable counting chambers: (i) the number of fields counted, (ii) the magnification factor of the microscope, (iii) the number of rows counted (row factor), and (iv) the dilution factor. The microscope factor should be calculated individually for each microscope [14]. The first one or two rows of the grid should be counted for samples with high sperm concentration, while the whole grid should be examined for samples with low concentration. Proper dilutions with phosphate buffered saline (PBS) solution should be made to facilitate the counting. Thus, sperm concentration is calculated according to the following formula:

$$\frac{\text{Total number of sperm counted}}{\text{Total number of fields counted}} \times \frac{\text{Microscope factor}}{\text{Total number of squares in the grid}} \times \frac{\text{Total number of rows in the grid}}{\text{Number of rows counted}} \times \text{Dilution factor}$$

Clinical Interpretation of Abnormal Concentration

For sperm concentration below 15×10^6/mL, the patient is classified as oligozoospermic [8]. Patients with suspected azoospermia should provide at least two samples on different days. The samples should be centrifuged to allow the deposition of any spermatozoa [17]. Azoospermia is usually classified as obstructive or non-obstructive with an extensive list of differential diagnoses including hypogonadism, testicular failure, CBAVD, and ejaculatory duct obstruction (EDO) [29]. Additional findings of low semen volume, low pH, and lack of fructose on semen analysis are suggestive of obstructive etiologies. Endocrine evaluation, including the assessment of serum follicle-stimulating hormone (FSH) and testosterone levels, which reflect germ cell epithelium and Leydig cell status respectively, may be useful in cases of oligozoospermia and non-obstructive azoospermia [23].

22.2.3.4 Undifferentiated Round Cells

Round cells include immature germ cells and leukocytes. Using the same preparation as for the assessment of sperm motility and concentration, the number of round cells can also be estimated under phase-contrast at a magnification of ×200. The number of round cells should be counted in the whole grid (100 squares) by applying the same rule of counting the cells that touch the upper and left edges and in five random fields [14]. Consequently, the total concentration of undifferentiated round cells is given by the following formula:

$$\frac{\text{Total number of round cells counted}}{\text{Total number of fields counted}} \times \frac{\text{Microscope factor}}{\text{Total number of squares in the grid}}$$

Concentrations of more than 1×10^6/mL round cells in semen are considered abnormal. An abnormally high number of immature germ cells may indicate testicular damage, while leukocytospermia is suggestive of inflammation. The characterization of leukocytes can be further confirmed by assessing the peroxidase activity with the Endtz test [14]. Moreover, an increased number of epithelial cells and the presence of large amounts of debris should be documented. Although the findings are regarded as abnormal, they are non-specific in diagnosis [8].

22.2.3.5 Morphology

The assessment of sperm morphology is crucial for the evaluation of sperm quality. This is particularly important for ART treatment because morphologically normal cells are selected and associated with a better reproductive outcome [44]. Morphology is the most complex and difficult sperm parameter to analyze and interpret as the shape of spermatozoa varies within an ejaculate of one individual, as well as between different samples from the same individual [45].

How Can It Be Evaluated?

Although the normal morphological features of human spermatozoa have been described, microscopic assessment of sperm morphology remains challenging. According to the WHO guidelines, the reference value for normal sperm morphology by the strict criteria is 4% [8]. A smear is prepared by placing a drop (5–15 µL) of the well-mixed sperm suspension onto a slide. After air-drying, slides should be

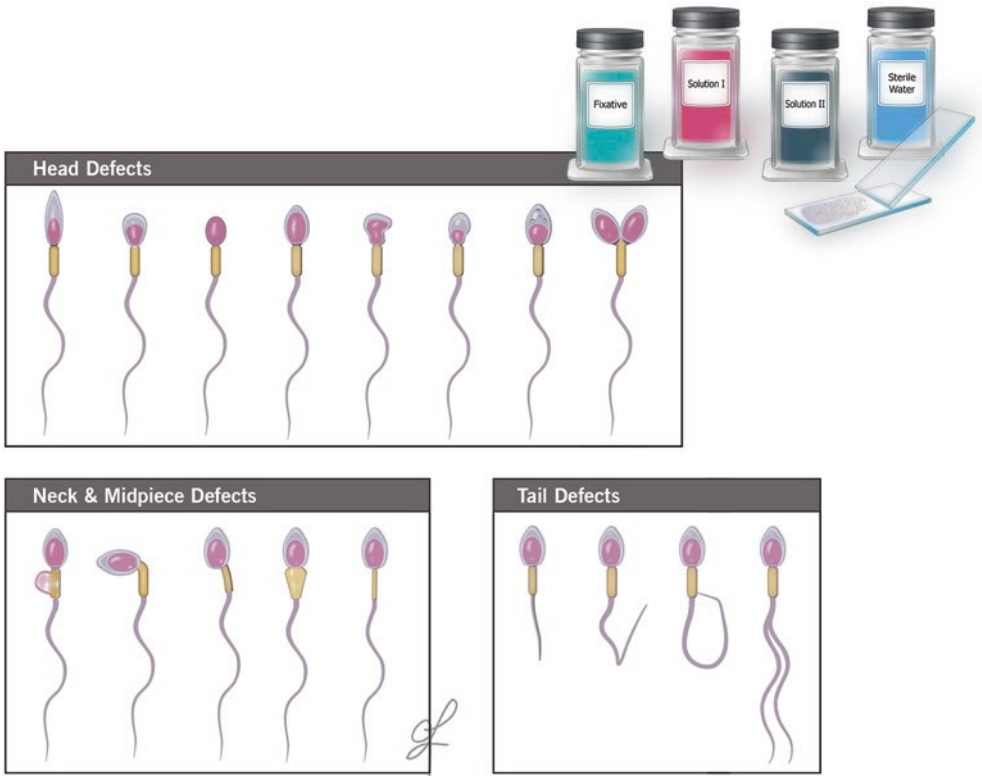

Fig. 22.3 Diff-Quik staining kit and common morphological defects of sperm head, neck and midpiece, and tail

fixed and stained by recommended staining methods: Papanicolaou, Shorr, or Diff-Quik. Diff-Quik (Fig. 22.3) is the most commonly used staining method at a clinical setting [8]. Although the slides can be directly examined under a microscope, the use of coverslip is recommended when long-term storage of the slides is required. Two hundred spermatozoa should be counted in each replicate (or twice in the same slide) in random fields using a bright field at ×1000 magnification with oil immersion to calculate the percentage of normality [8].

Clinical Interpretation of Abnormal Morphology

Abnormal sperm morphology (<4%) is classified as teratozoospermia. The origin and impact of some morphological abnormalities are still poorly understood. However, certain adverse sperm morphologies are attributed to abnormal spermatogenesis or sperm maturation, and suggestive of certain pathologies. Abnormal morphology may have an impact on chromatin condensation, acrosome reaction, sperm motility, and apoptosis/necrosis. Specific morphological defects, such as globozoospermia, sperm macrocephaly syndrome, multiple tail abnormalities, or headless spermatozoa, may be associated with genetic abnormalities [46].

22.3 Adjunct Tests for Semen Analysis

22.3.1 Vitality

The evaluation of sperm vitality (or viability) differentiates live or dead immotile spermatozoa. Assessment of sperm vitality is considered an adjunct test because it is recommended for samples with low sperm motility [8]. Analysis of sperm vitality is based on the integrity of sperm cell membrane. The most common methods include eosin-nigrosin staining technique and the hypo-osmotic swelling test. The reference value for normal vitality is ≥58% for both testing methods [8]. The presence of a large proportion of vital but immotile cells may be indicative of structural defects in the flagellum [47], whereas a high percentage of non-viable cells (necrozoospermia) may suggest epididymal pathology [48].

22.3.1.1 Eosin-Nigrosin Staining

Eosin-nigrosin staining is one of the most common methods to assess vitality. A drop of sperm suspension is mixed with two drops of eosin and nigrosin solutions (Fig. 22.4a) and a smear is prepared onto a slide and covered with a coverslip [49]. Eosin will penetrate the nucleus of membrane-damaged spermatozoa by staining non-viable sperm light-to-dark pink, while intact living cells will have the ability to exclude the dye and appear white (Fig. 22.4a). The background purple color of nigrosin facilitates the analysis. The examination of the slides under the microscope should be made using a bright field at ×1000 magnification with oil immersion. A total of 200 cells should be counted in different random fields and in replicate slides, and the percentage of viable cells can be calculated [50].

22.3.1.2 Hypo-osmotic Swelling Test

The hypo-osmotic swelling test is based on the principle that only living cells with intact membranes will swell under hypo-osmotic conditions (150 mOsmol/L). The influx of fluid into viable cells is reflected by the curling of sperm tails

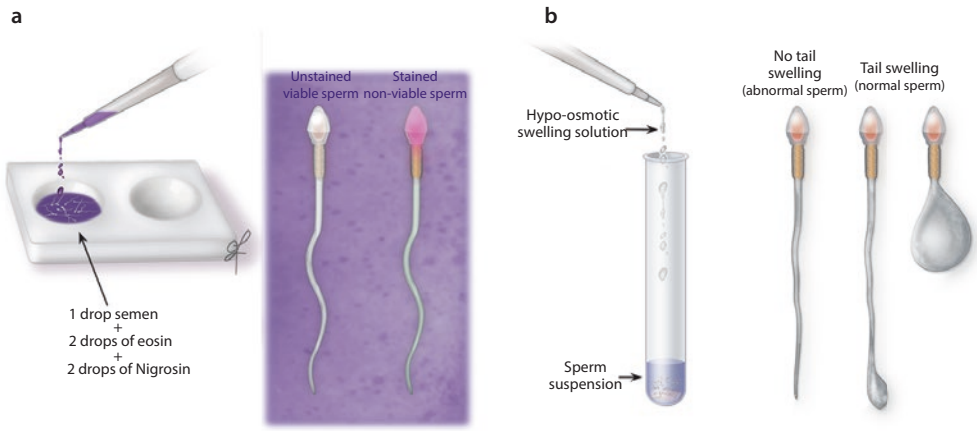

Fig. 22.4 Sperm vitality tests. Eosin-nigrosin staining **a** and hypo-osmotic swelling **b**

(Fig. 22.4b) [51]. One of the advantages of this test is that it does not kill the spermatozoa which allows the utilization of those spermatozoa for ART [52]. In this method, the sperm sample should be mixed with the hypo-osmotic solution and incubated at 37 °C for 30 min (5 min if the sample is going to be used for ART). After incubation, a wet preparation should be made to assess the sample under a phase-contrast microscope (×200 or ×400 magnification). A total of 200 spermatozoa should be counted and classified as dead or alive [8].

22.3.2 Endtz Test

Granulocytes (polymorphonuclear leukocytes) are the most abundant type of leukocytes in human ejaculates and neutrophils are the most common subtype. A simple histochemical test, also referred to as Endtz test, is used to detect peroxidase within neutrophils, thus allowing the screening of granulocytes in the semen. This test relies on the ortho-toluidine staining of peroxidase-positive cells and should be performed when an abnormal concentration of round cells is detected ($>1 \times 10^6$/mL) [53]. However, Endtz test detects neither peroxidase-free leukocytes (e.g., lymphocytes, macrophages, or monocytes) nor those granulocytes that have already released their granules [8].

A 20 µL drop of semen is mixed with the same volume of PBS and the double volume of freshly prepared working Endtz solution. The mixture is incubated for 5 min at room temperature and protected from light. After incubation, 5 µL of the mixture is placed into a Makler chamber and analyzed under a bright field optic (×200 magnification). The whole grid (100 squares) of the counting chamber should be analyzed by counting all the dark brown-stained peroxidase-positive cells [54].

A concentration of leukocytes $\geq 0.2 \times 10^6$/mL in a semen sample is generally considered Endtz positive [54]. However, a higher reference value is usually acceptable for routine semen analysis, and an Endtz positive sample is defined with a concentration of leukocytes $\geq 1 \times 10^6$/mL [8]. Abnormal leukocyte count is a common finding in subfertile men and may reflect an underlying inflammatory/infective ethology [55]. The incidence of leukocytospermia in infertile men varies between 3% and 23% and has been correlated with clinical and subclinical genital infections, elevated anti-sperm antibody levels, and high levels of reactive oxygen species (ROS) [56, 57].

22.3.3 Fructose Test

Fructose in semen is a marker for seminal vesicles function and is usually performed to localize the level of obstructive azoospermia in men with low ejaculate volume. The concentration of fructose can be evaluated by a biochemical assay, which is based on the ability of fructose to form a colored complex with the indole [58]. This method should be performed after concluding the routine semen analysis since it requires the centrifugation (1000 × g for 10 min) of the semen to separate seminal plasma from spermatozoa. A 5 µL drop of seminal plasma should be diluted in 50 µL of distilled water and then mixed with $ZnSO_4$ (63 µmol/L) and NaOH (0.1 mol/L) to allow deproteinization. After incubation of the mixture for 15 min at room temperature, the sample should be centrifuged at 8000 × g for 5 min. Then, 50 µL of the supernatant is mixed with the same volume of indole reagent and 5 mL of hydrochloric acid (HCl 32% v/v) and incubated at 50 °C for 20 min, followed by 15 min of cooling on ice. The absorbance of the formed complex is measured with a spectrophotometer at 470 nm and the concentration of fructose in the sample is calculated according to a standard curve. The lower reference limit for fructose defined by the WHO is 13 µmol per ejaculate [8]. A low content of fructose in seminal plasma together with a low semen volume and low pH supports the diagnosis of obstructive azoospermia [59]. Low fructose is also characteristic of CBAVD or in men with partial retrograde ejaculation [60, 61].

22.4 Computer-Assisted Sperm Analysis (CASA)

Computer-Assisted Sperm Analysis (CASA) systems have been developed in the last three decades with the main objective of reducing the subjectivity of the current semen assessment technique and possibly decreasing intra- and

inter-observer variability [62]. These systems also aim to reduce the labor-intensive and time-consuming nature of the standard techniques. However, the complexity of semen analysis which includes multiple parameters and a wide spectrum of microscopic skills poses difficulties in the development of automated computer systems. Currently, many CASA manufacturers are using different software and principles. Most of these systems establish a centroid for each spermatozoon and evaluate cell motion based on centroid trajectory, while others focus on bulk movement of the sperm population. Another type of system uses continuous images to correlate properties of each pixel in one image with temporal fluctuations of the signal from the same pixel to the subsequent image [62]. This variety of algorithms together with the increasing sophistication of hardware complicates the comparison of results between different CASA systems. Nevertheless, many laboratories have included CASA in the routine assessment of sperm parameters mainly as a control for the comparison to standard technical analysis, rather than as a stand-alone replacement technique [63]. There is still a lack of consensus regarding the clinical application of this technology, especially due to the low number of studies supporting its accuracy and correlation with reproductive outcomes.

22.4.1 Which Semen Parameters Can Be Evaluated by CASA?

The majority of currently marketed CASA systems allow the analysis of sperm concentration, vitality, and motility, while only a handful of systems available in the market have incorporated the computer-aided sperm morphometric assessment (CASMA) tool [63]. Sperm concentration is an objective parameter of semen analysis. The principle of evaluating sperm concentration is the same between manual and computer counting thus resulting in comparable sperm counts [64]. However, the computed-assisted method is faster and allows the analysis of more samples in a shorter time. The accuracy of sperm concentration analysis by CASA is highly dependent on the sperm counting chamber that is specifically affiliated to it [65]. Recent CASA systems have improved the accuracy of sperm concentration analysis by the incorporation of DNA fluorescent-staining. This allows a better distinction of spermatozoa from non-sperm particles by using a fluorescent microscope [66]. Moreover, a few systems incorporated mathematical algorithms specific for tail-detection to avoid overestimation of sperm concentration by counting non-intact spermatozoa or debris. However, the precision of the measurement by CASA decreases outside an optimal range of sperm concentration between 2 and 50×10^6/mL [67]. The same fluorescent principle is used by some CASA instruments for the determination of sperm vitality. Recently, some CASA manufacturers are trying to implement the assessment of eosin-nigrosin staining automatically in order to reduce the costs associated with fluorescence microscopy [68].

Sperm motility and morphology involve a subjective assessment by the technician during standard semen analysis, and one way to overcome this issue is by defining objective criteria for assessment of sperm motility and morphology. The assessment of sperm motility with CASA is based on system-specific algorithms to measure the sperm kinematics, i.e., the characteristics that describe sperm movement, as opposed to the proportion of motile cells. Sperm kinematics is reflected by three parameters: sperm motion velocity, velocity ratios, and wobble (Table 22.2) [63]. Therefore, the assessment of multiple objective criteria by CASA systems may be particularly helpful when assessing sperm progressive motility. The information allows more detailed categorization of sperm motility into four grades: (i) Grade 4, rapidly progressive cells; (ii) Grade 3, slowly progressive cells; (iii) Grade 2, weakly twitching cells (non-progressive); and (iv) Grade 1, immotile cells, compared to the three-grade system recommended by the WHO guidelines for manual assessment. At least 100 spermatozoa should be counted in a total of 5 random fields [14]. After calculating the percentage for each category, the predominant grade should be reported. Subclassifications of progressive motility into rapid and slow subgroups by CASA would not be feasible with manual assessment without significant bias [69].

The use of CASMA tool for sperm morphology allows the classification of a spermatozoon as normal or abnormal according to the dimensions of the head and midpiece, head ellipticity, and regularity, and they can identify the acrosome area by a staining method [8]. The multitude of unpredictable shapes and defects that spermatozoa may present in the sample, as well as its similarity with other non-sperm cells represents the major obstacle in efficient assessment of sperm morphology by automated systems.

22.4.2 Advantages and Disadvantages of CASA

CASA systems have the potential to revolutionize semen analysis. The technique decreases subjectivity during standard manual semen analysis. The system also allows more rapid analysis of a larger sample which will reduce sampling error. More importantly, introduction of novel concepts in assessment of motility by kinematics may better reveal the overall quality of sperm. CASA also provides advanced image tools that allow the recording of pictures and videos throughout the analysis (Fig. 22.5), which may be helpful for standardization and quality assurance. This would also give the possibility of data re-analysis whenever needed by a clinician while establishing a diagnosis or selecting an ART treatment.

The limited number of parameters that can be measured by CASA compared to standard procedures represents a major pitfall of the system. The cost-effectiveness of using the CASA system in Andrology laboratories is doubtful when a significant portion of macroscopic and microscopic evaluation of semen samples has to be done manually. In addition, the equipment requires constant supervision and calibration,

Table 22.2 Characteristics of sperm kinematics evaluated by the CASA instruments [63]

Characteristics	Parameters	Abbrev	Meaning	Details
Sperm motion velocity	Curvilinear velocity	VCL (μm/s)	Time-averaged velocity of a sperm head along its actual curvilinear path	Highest value among sperm motion velocity parameters
	Straight-line velocity	VSL (μm/s)	Time-averaged velocity of a sperm head along the straight line between its first detected position and its last, reflecting the net space of a sperm's forward motility during the observation period	Lowest value among sperm motion velocity parameters
	Average path velocity	VAP (μm/s)	Time-averaged velocity of a sperm head along its average path, which is determined by smoothing the curvilinear trajectory according to specific algorithms	VAP is almost identical to VSL when the sperm motion trajectory is very regular and linear, and it is much higher than VSL when the sperm motion path is irregular
Sperm velocity ratio	Linearity	LN	VSL/VCL ratio	Linearity of a curvilinear path
	Straightness	STR	VSL/VAP ratio	Linearity of the average path
	Wobble	WOB	VAP/VCL ratio	Oscillation of the actual path in comparison with the average path
Sperm wobble	Amplitude of lateral head displacement	ALH (μm)	Magnitude of lateral displacement of a sperm head about its average path	Can be expressed as a maximum or an average of such displacements
	Beat-cross frequency	BCF (Hz)	Average rate at which the curvilinear path crosses the average path	Useful parameter assessing the changes in wobble way of a sperm flagellum
	Mean angular displacement	MAD (degrees)	Time-averaged absolute values of the instantaneous turning angle of the sperm head along its curvilinear trajectory	Very sensitive to the sampling rate

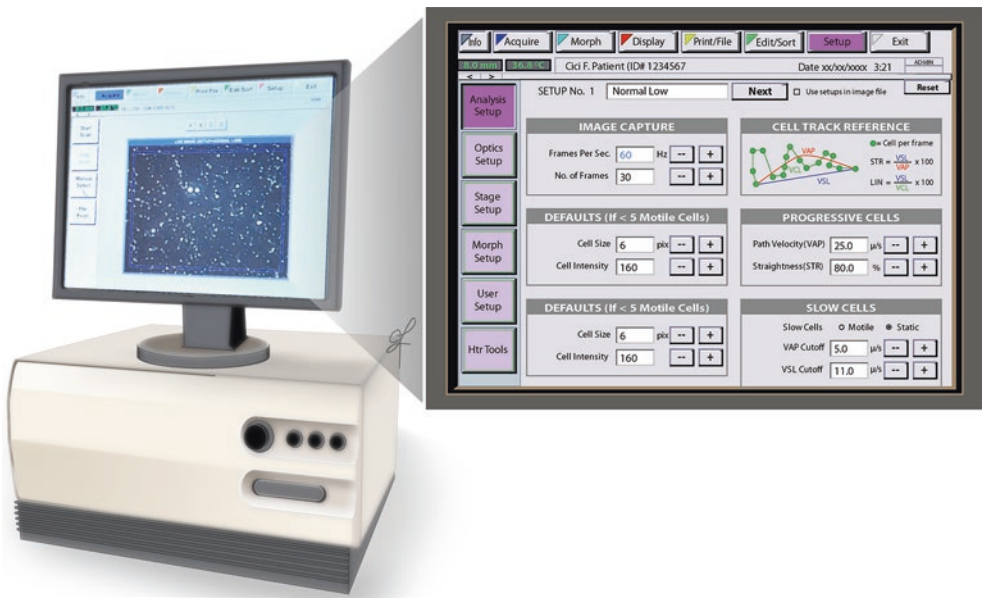

Fig. 22.5 Representative image of a computer-assisted semen analysis (CASA) system and display of the obtained results

as well as specific supplies such as counting chambers and well-trained personnel. There is currently a huge variety of hardware and software available in the market. The lack of standardization of CASA systems makes the comparison of results among different CASA instruments impossible. Another drawback is the insufficient robustness of CASA as a stand-alone technique, which is mostly due to concomitant biological factors and technical errors. Biological factors

including semen hyperviscosity, presence of other cell types, the highly pleomorphic nature of sperm, and sperm agglutination may significantly hinder CASA interpretation without prior specimen preparation. Technical errors due to improper sample preparation and loading into the chamber, excessive time taken from sample preparation to analysis, and lack of internal quality control and training may represent another source of error during CASA semen analysis [68]. There is also a lack of accuracy in CASA semen analysis due to the limited capability of the available software to distinguish spermatozoa from other objects of similar size such as round cells, cytoplasmic droplets, and debris, as well as difficulties inherent to sperm clumping.

Furthermore, only a small number of studies provide evidence for the significance of sperm analysis through CASA in the prediction of reproductive outcomes. A significant association has been observed between the CASA analysis of sperm concentration and progressive motility with the fertilization rates and time to conception [70, 71]. Moreover, CASMA tools were also reported as significant predictors of in vitro fertilization rates [72] and natural pregnancy in subfertile couples [67]. More studies are needed to clarify the significance of CASMA automated tools using larger samples. Currently, there is no consensus on the clinical utility of CASA systems for diagnostic purposes. Further studies on standardization of reference values and proper training of technicians are essential for better credibility and acceptance of CASA semen analysis in clinical practice.

22.5 Recent Advances in Semen Analysis

Recent technological advances led to the development of some new approaches of semen analysis, particularly the home testing methods, in an attempt to increase compliance rates and privacy [73]. The technology possibly reduced the reluctance of patients to seek medical fertility advice and minimize embarrassment in collecting a semen sample at a clinical laboratory. Moreover, it also allows immediate testing upon sample collection, thereby reducing potential errors due to transportation and handling. One of the products which are commercially available with promising accuracy is the SpermCheck Fertility Test. This test is based on a color change antibody reaction that allows a quick estimation of sperm concentration in the ejaculate. It is an easy-to-use stepwise method that gives a positive result when sperm concentration is at least 20×10^6/mL and negative result below that limit [74]. Similar tests with the same principle are available, such as Fertell and SwimCount devices, to estimate total motile count and a paper-based test that estimates concentration, motility, and viability [73]. Interestingly, several smartphone-based approaches have been proposed such as the Trak device or the YO Sperm Test. Trak is provided as a package including a centrifugation device and disposable microfluidic chambers which is sufficient to perform four tests. This device allows the classification of sperm concentration as low ($<15 \times 10^6$/mL), moderate (15×10^6/mL), or optimal (55×10^6/mL) for conception, and the provided smartphone app allows users to enter data and track their sperm count. The YO sperm test includes a device that can be directly connected to a smartphone to see the results within the app itself. It allows the estimation of the concentration of motile sperm by using the phone's camera and flash to record a video of the sperm in the sample [73]. Smartphones are a worthy testing platform since they are commonly used; have computing power, internet access, and high-resolution cameras; and allow data storage [75]. Their use may serve as a screening tool for patients before seeking medical assistance for further confirmatory testing and it may increase the awareness for a possible fertility problem. In contrast to traditional laboratory tests, the easy repeatability of the tests at a low cost allow the recording of changes in sperm parameters over time and may serve as a convenient means to monitor treatment response. Although these promising tests have shown high percentage of accuracy in pilot studies, they are also associated with some drawbacks inherent to their novelty. One of the main issues is that most of these home testing devices can only provide information on a single or a few sperm parameters at a time.

22.6 Conclusion

The correct interpretation of semen analysis results is based on a combination of multiple sperm parameters together with clinical information. Coexistence of multiple altered seminal parameters signifies the probability of sperm dysfunction and is associated with male infertility. Therefore, proper handling of sample and standardization of semen analysis is of utmost importance for diagnosis and evaluation of infertile men. The development of automated systems such as CASA aims to overcome at least some of the current limitations of semen analysis by providing objectivity, precision, and reproducibility compared to standard manual analysis. However, questionable efficiency, lack of standardized guidelines, inconsistency between different equipment, and high costs of CASA systems limit its current clinical application. Finally, home testing approaches have shown promising accuracy and may be adopted as a valuable tool. The technology may be a cost-effective self-screening test before seeking medical assistance.

Review Questions (MCQ Format)

1. Which of the following statements concerning semen analysis is incorrect?
   (a) Semen analysis is the most widely used laboratory test in male fertility assessment.
   (b) The World Health Organization manual provides reference values for semen analysis.
   (c) 7–10 days of abstinence is suggested before semen sample collection.
   (d) Evaluation of specimen should start after liquefaction.

2. What is the type of information provided by traditional semen analysis?
   (a) Information regarding the patient's semen quality
   (b) Information about the patient's fertilizing potential
   (c) Information about the function of the accessory glands
   (d) All of the above

3. Which of the following parameters is not part of routine microscopic evaluation of a semen sample?
   (a) Sperm concentration
   (b) Sperm motility
   (c) Sperm morphology
   (d) Sperm vitality

4. Which of the following sentences is correct about routine semen analysis?
   (a) A single parameter can discriminate between infertile and fertile men
   (b) Three altered parameters indicate a diagnosis of infertility
   (c) Routine semen analysis by itself is not sufficient to establish fertility status
   (d) All the sentences are incorrect

5. Which of the following statements concerning computer-assisted sperm analysis (CASA) systems is correct?
   (a) Standard manual semen analysis will gradually be replaced by CASA.
   (b) Sperm kinematics is a novel concept in assessment of sperm motility by CASA.
   (c) CASA systems report semen parameters in the same way as standard semen analysis.
   (d) Sperm morphometric assessment provides accurate results.

6. What are the factors hindering the clinical implementation of computer-assisted sperm analysis (CASA) systems?
   (a) Lack of standardization and inability of comparison between systems
   (b) Ability to record pictures and videos during the analysis
   (c) Increased objectivity relative to standard procedures
   (d) Analysis of many samples in a short time

7. What are the advantages of smartphone-based approaches in the assessment of infertile men?
   (a) First screening before seeking medical advice
   (b) Easiness to perform the test and quickness of the results
   (c) Low-cost and high correlation with laboratory results
   (d) All of the above

8. Which of the following statements concerning home testing approaches in semen analysis is correct?
   (a) Home testing kits are not commercially available currently.
   (b) The approach allows convenient sample collection and immediate analysis at home.
   (c) Home testing devices provide assessment of multiple semen parameters.
   (d) The use of home testing kits requires special training.

Answers

1. (c)
2. (d)
3. (d)
4. (c)
5. (b)
6. (a)
7. (d)
8. (b)

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Sperm Assessment: Novel Approaches and Their Indicative Value

Tania R. Dias, Chak-Lam Cho, and Ashok Agarwal

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Learning Objectives

- Limitations of conventional semen analysis
- Laboratory semen assessment beyond routine procedures
- Assessment of prooxidant-antioxidant semen profile: reactive oxygen species (ROS) levels, total antioxidant capacity (TAC), and oxidation-reduction potential (ORP)
- Methods to evaluate sperm DNA fragmentation
- Assessment of sperm fertilization capacity: acrosome reaction and sperm mitochondrial function
- Tests for antisperm antibodies
- Clinical value of advanced semen and sperm assessment

23.1 Introduction

Conventional semen analysis is the essential basic investigation for male fertility assessment during the evaluation of an infertile couple [1]. However, the enthusiasm in male factor infertility has diminished since the introduction of intracytoplasmatic sperm injection (ICSI) in 1992. Sperm without the ability to penetrate the ovum could be directly injected into the oocyte cytoplasm to achieve fertilization by ICSI. This revolutionized the approach of reproductive medicine toward assisted reproductive technology (ART) and shifted the emphasis away from evaluation of infertile men, as the most severe form of male infertility could be possibly bypassed by ICSI [2]. However, the possible threat to the offspring's health remains a concern, since defective sperm may fertilize an ovum by ICSI [3]. The identification and correction of male factors is essential to improve the reproductive outcomes of infertile couples.

Standard semen analysis has its own drawbacks in establishing the fertility status of a man and is prone to errors. Although the equipment and techniques have changed over time, the basic principles of semen analysis remained the same over the years [4]. Multiple altered semen parameters are found in infertile men, but the findings are usually nonspecific for diagnostic purposes [5]. Abnormal semen parameters cannot accurately predict infertility. On the other hand, a significant proportion of infertile men have apparently normal semen parameters and are classified as idiopathic male infertility [6]. The imprecision of standard semen analysis to predict the reproductive outcome is essentially due to the inherent biological variability between ejaculates [7] and subjectivity of human manual analysis [8]. Despite all the efforts of World Health Organization (WHO) to standardize semen analysis procedures and define reference values, different laboratories continue to use different techniques and guidelines [9]. The pitfalls of conventional semen analysis led to the exploration of novel approaches. Evaluation of sperm function represents one of the most important research targets. Normal sperm function involves multiple interrelated factors that are essential to achieve fertilization, pregnancy, and embryonic development. The attempt to detect abnormal sperm function has prevailed for decades without success and constitutes one of the major challenges to fertility specialists. Some sperm function tests have long been developed to assess sperm-oocyte penetration, capacitation, acrosome reaction, and antisperm antibodies with the aim to overcome the limitations of conventional semen analysis. However, none of the tests has been widely implemented in clinical practice and only a few remained after the emergence of ICSI. Currently, acrosome reaction testing, sperm mitochondrial activity assay, and antisperm antibody tests are utilized by some of the andrology laboratories.

The novel concept of OS has been identified as a major cause for male infertility. The clinical significance of laboratory tests to evaluate OS by measuring reactive oxygen species (ROS), total antioxidant capacity (TAC), and oxidation-reduction potential has been investigated. Sperm DNA fragmentation represents one of the consequences of OS and a target for laboratory assays. The negative implication of high sperm DNA fragmentation to reproductive outcomes has been demonstrated by various studies. However, emerging evidence supports the ability of these tests to provide additional information and better discriminate between infertile and fertile men. The wide clinical application of the tests is still predestined to further research. This chapter will cover the principles and clinical value of currently available laboratory tests beyond routine semen analysis, as well as future directions for the development of new approaches.

23.2 Assessment of Prooxidant-Antioxidant Semen Profile

23.2.1 Reactive Oxygen Species

23.2.1.1 What Are Reactive Oxygen Species?

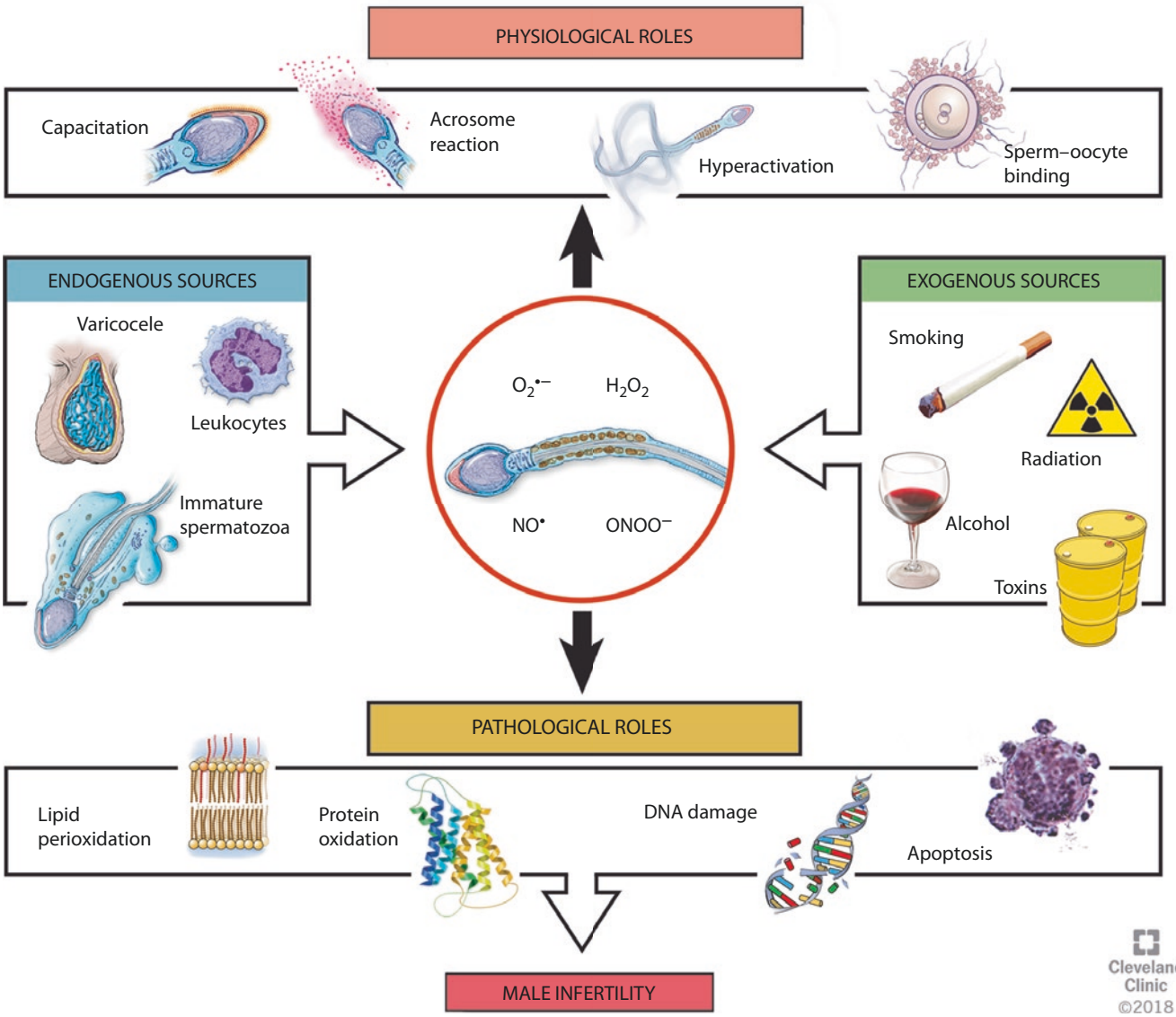
The term ROS refers to by-products of the normal metabolism of oxygen [10]. They can be free radicals, which are unstable and highly reactive compounds due to the presence of at least one unpaired electron in an outer orbital. Examples of free radicals in biological systems include superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$). However, some non-radical oxygen derivatives are also included in the ROS category such as singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and hydrogen hypochlorite ($HOCL^-$). These non-radical compounds do not contain unpaired electrons, but they are still potentially reactive and can be converted to radical ROS [11]. Another important type of ROS prooxidant includes those containing nitrogen in their constitution, sometimes called as reactive nitrogen species (RNS). Nitric oxide ($NO^{\bullet}$) and nitrous acid (HNO_2) are examples of radical RNS, while nitrogen dioxide (NO_2) and dinitrogen tetraoxide (N_2O_4) are non-radical RNS [12]. Throughout this chapter, the abbreviation ROS will include all the aforementioned types.

The main sources of ROS in semen are immature or abnormal spermatozoa and leukocytes. ROS are physiologically generated by mitochondria during energy production [13]. Although

most of the oxygen consumed by the mitochondrial electron transport chain is reduced to water, a small proportion is converted to ROS. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase on the plasma membrane is also another source of cellular ROS [14]. Pathological conditions such as varicocele or other exogenous factors including cigarette, radiation, or drugs are major stimuli for ROS overproduction.

23.2.1.2 How Do Reactive Oxygen Species Affect Sperm Quality?

ROS have a physiological role and are essential for the regulation of cell signaling pathways, enzymatic activities, and immune defenses [15]. In addition, ROS play an important role in sperm fertilization, as they are one of the triggers for hyperactivation, capacitation, acrosome reaction, and sperm-egg fusion (◘ Fig. 23.1) [16]. Excessive ROS that are not used for biological processes are stabilized or eliminated by antioxidant defenses, so that the prooxidant-antioxidant homeostasis can be maintained. However, OS is established when excessive ROS production overwhelms the capacity of cellular antioxidant system, or when antioxidant reserves are abnormally low. OS can compromise sperm quality via impaired spermatogenesis, defective sperm function, or even apoptosis [17]. Among the most common ROS produced in sperm are superoxide anion, hydrogen peroxide, nitric oxide, and peroxynitrite. ROS can "steal" an electron from a target molecule to attain stability. The molecule that loses the electron becomes a free radical itself, which will also take an electron from a nearby molecule. This will trigger a cascade of reactions that, if uncontrolled, will result in severe cellular damages and even cell death. Proteins, lipids, and DNA are the core targets of ROS, resulting in protein oxidation, lipid peroxidation, and/or oxidation of DNA bases, respectively [11]. Spermatozoa are particularly susceptible to oxidative



◘ **Fig. 23.1** Physiological versus pathological roles of reactive oxygen species (ROS) in spermatozoa and the main endogenous and exogenous ROS sources

damages due to their limited intracellular defense system. During spermiation, most of the sperm cytoplasm is removed, thus removing most of the antioxidant defenses as they are mostly present in the cytoplasm. Besides, the sperm plasma membrane is rich in polyunsaturated fatty acids (PUFAs), which are prone to oxidation in the presence of ROS, generating lipid peroxyl radicals. Those radicals contribute to the formation of hazardous adducts, such as the 4-hydroxynonenal (4-HNE) and thiobarbituric acid reactive species (TBARS), which initiate a chain reaction of free radical generation described as lipid peroxidation. Consequently, there is a loss of fluidity in spermatozoa membrane that results in impaired sperm motility and fertilization failure [18]. Sperm proteins constitute another target of ROS attack. Oxidation of proteins by ROS leads to the addition of carbonyl groups to their side chain. This alteration in the structural conformation of proteins can result in the inactivation of key enzymes involved in sperm function. The effects on sperm DNA will be discussed later in this chapter.

23.2.1.3 Methods to Measure Reactive Oxygen Species

Several methods can be used to evaluate ROS generation in semen or specifically in spermatozoa. The short half-lives of ROS constitute the major obstacle in the direct measurement of ROS. Alternatively, ROS can be measured indirectly by the assessment of end-products generated after ROS attack to semen/sperm biomolecules. Fresh samples are preferred for ROS measurement because sample storage (at −80 °C or cryopreservation) has been associated with a higher production of ROS [19, 20].

Chemiluminescence

One of the most common techniques to measure ROS levels in semen is by chemiluminescence [21]. This method is based on the production of light through the reaction between a probe and ROS. The emitted light is converted to an electrical signal (photon) by a luminometer and the ROS levels in the sample are calculated as relative light units (RLU) per second (Fig. 23.2) [22]. This method allows a real-time monitoring by a computer system. Two main types of probes can be used: luminol for both intracellular and extracellular ROS and lucigenin for extracellular ROS. Luminol is an uncharged membrane permeable molecule that can react with a variety of ROS, including $O_2^{\bullet-}$, H_2O_2, OH, and peroxynitrite. Lucigenin carries a positive ionic charge, which makes it membrane-impermeable and it reacts mainly with $O_2^{\bullet-}$ [23]. The resulting value is then normalized to the sperm concentration in the semen to estimate ROS generated by spermatozoa. Recently, a reference value of <102 RLU/s/million sperm was proposed as normal for ROS levels in whole ejaculates [24]. There are multiple confounding factors which may affect the result of chemiluminescence assays, including sample volume, time of analysis, viscosity of the sample, concentration of reactants, reagent injection, temperature, and background luminescence [24]. Advantages and disadvantages of the test are presented in Table 23.1.

Fluorescence: Flow Cytometry

The intracellular levels of ROS can be measured by flow cytometry using non-fluorescent probes. 2,7-Dichlorofluorescein diacetate (H_2DCFDA) is a stable cell-permeable probe that de-esterifies in the presence of H_2O_2 to form a fluorescent 2,7-dichlorofluorescein (DCF) product. Other ROS such as peroxynitrite, hypochlorous acid, and OH• can also oxidize this probe and may significantly contribute to the positive signals observed in defective spermatozoa [25]. Another probe, dihydroethidium (DHE), can be oxidized mainly by superoxide ions and to a much lesser extent by other ROS [26], resulting in the hydroxylation at the 2-position forming 2-hydroxyethidium with red florescence emission at 488 nm, which will stain the mitochondrial and nuclear DNA [25]. The oxidation of these dyes results in fluorescent derivatives that can be detected by the flow cytometer. These probes can be used along with vitality (SYTOX green) or apoptotic (propidium iodide)

Fig. 23.2 Luminometer and real-time monitoring of reactive oxygen species (ROS) levels through a connected computer

Table 23.1 Advantages/disadvantages of the available methods to measure reactive oxygen species (ROS)

Method	Type of sample	Principle	Advantages	Disadvantages
Chemiluminescence	Whole ejaculate	Measures ROS production in real time using specific probes: luminol/lucigenin	Robust High sensitivity High specificity Measure intracellular and extracellular ROS Reproducible	Expensive Time-consuming Limited half-lives of the probes and ROS Requires large sample volume
Fluorescence	Whole ejaculate	Measures the fluorescent signal emitted by the oxidation of non-fluorescent probes by ROS	A large number of cells can be analyzed High specificity for intracellular ROS Accurate Allow discrimination between different cell types Allow analysis of ROS-generating live cells	Requires sophisticated and expensive hardware The results do not quantify the target ROS
TBARS	Whole ejaculate Seminal plasma	Measures lipid peroxidation by detecting MDA-TBA complex formation	Simple Rapid Inexpensive	Nonspecific for any ROS type Indirect method Requires rigorous standards
NBT	Whole ejaculate Leukocytes Spermatozoa	Estimates ROS production by NBT reduction to formazan	Simple Low cost Readily available Allow cellular-specific estimation	Lack of specificity due to cross-reactivity with oxidoreductases

ROS reactive oxygen species, *TBARS* thiobarbituric acid reactive substances, *MDA* malondialdehyde, *TBA* thiobarbituric acid, *NBT* nitroblue tetrazolium

markers to identify the percentage of live cells generating ROS. The results of ROS levels are interpreted as percentage of fluorescent spermatozoa. Fluorescent techniques have a higher specificity, accuracy, sensitivity, and reproducibility for intracellular ROS detection, compared to chemiluminescence (Table 23.1). Samples tested negative by chemiluminescence may have high intracellular H_2O_2 generation that can be detected by flow cytometry [27]. Global ROS measurement by chemiluminescence was positively correlated with the estimates of overall intracellular levels of H_2O_2 and of peroxynitrite assessed by flow cytometry [25]. Flow cytometry allows the discrimination of the contribution of each cell type to the ROS levels, which may be of great value at a clinical setting [25, 27].

Thiobarbituric Acid Reactive Substances (TBARS)

Thiobarbituric acid reactive species (TBARS) are formed as a by-product of lipid peroxidation and can be detected by a reaction with thiobarbituric acid (TBA). One of those TBARS products is malondialdehyde (MDA), which forms a pink complex when reacting with TBA at high temperature (90–100 °C) and low pH [28]. MDA-TBA complex can be colorimetrically measured at 530–540 nm or fluorometrically at an excitation/emission wavelength of 530/550 nm. Results are expressed as nmol MDA 10^{-7} sperm [29]. MDA is just one of the products generated by lipid peroxidation and it is not exclusively generated through this process. Some authors have established the normal limit value of TBARS method for sperm (0.0287 ± 0.0162 nmol/10^8 sperm) and seminal plasma (0.65 ± 0.17 nmol/mL) [30].

Nitroblue Tetrazolium (NBT)

Nitroblue tetrazolium (NBT) is a yellow water-soluble nitro-substituted aromatic tetrazolium compound that forms an insoluble purple-black product called formazan when it is reduced. When isolated cells such as spermatozoa or leukocytes are incubated with NBT solution, they take up the tetrazolium salt into their cytoplasm and convert it to water-insoluble purple-black formazan crystals by the activity of superoxide anions [23]. Those intracellular formazan crystals can be visualized under an optical microscope. The cells stained with NBT are NBT-positive cells, and there is a relation between the NBT-positive cells and the levels of ROS in the same suspension [31]. Alternatively, a cell suspension (whole ejaculate) can be combined with NBT solution, and the formed formazan crystals can be released using a solubilization reagent and quantified spectrophotometrically [32]. However, the specificity of this test has been questioned (Table 23.1), hindering its clinical use. In theory, not only superoxide anion but any enzyme capable of reducing NBT will result in the formation of formazan. The same response can be generated by a number of oxidoreductases present in seminal plasma using alternative electron donors [33].

23.2.2 Antioxidant Capacity

23.2.2.1 Which Are the Antioxidant Defenses in Semen?

Antioxidants are compounds with the ability to scavenge ROS or act as cofactors for the cellular enzymatic antioxidant defenses, thus preventing OS-induced damages [34]. Most of the sperm cytoplasm and organelles are removed during spermiation; thus, the sperm endogenous defense system becomes limited. Sometimes the cytoplasm removal is not complete, and spermatozoa retain a residual cytoplasmic droplet, which contains ROS-promoting enzymes [35]. Spermatozoa are then dependent on the protection provided by epididymal fluid and seminal plasma during their maturation processes. These fluids are endowed with enzymatic antioxidants, such as superoxide dismutase, catalase, glutathione peroxidase, glutathione transferases, peroxiredoxins, and thioredoxins, and non-enzymatic antioxidants, including vitamin C (ascorbic acid), vitamin E (tocopherol), and zinc [36, 37]. The non-enzymatic antioxidants constitute the main defense system to scavenge excessive ROS as they account for approximately 65% of the total antioxidant capacity (TAC) of the seminal plasma [38].

23.2.2.2 Methods to Measure Total Antioxidant Capacity

Measuring TAC in semen can give important information regarding the seminal environment and sperm protection. Many studies showed that decreased antioxidant capacity is associated with impaired sperm function [39, 40]. TAC can be measured by evaluating the reducing ability of various seminal antioxidants against an oxidative reagent such as hydrogen peroxide and measuring the effect on the substrate. This will serve as an OS measurement from another facet in addition to ROS production. Reduced TAC will increase the susceptibility to ROS-induced damages and may contribute to OS. Measuring the TAC may provide more relevant biological information compared to that obtained by the measurement of individual components, as it considers the sum of endogenous and food-derived antioxidants present in plasma or body fluids. Different modes of action of antioxidants led to the development of hydrogen atom transfer (HAT) and single electron transfer (SET) assays. HAT-based assays measure the capability of an antioxidant to quench free radicals (generally, peroxyl radicals) by H-atom donation. SET-based assays simulate the antioxidant action by a redox potential probe. The capacity of an antioxidant to reduce the oxidant result in a color change that can be spectrophotometrically quantified [41]. If possible, fresh samples should be used since antioxidants are prone to oxidation during sample storage.

Ferric Reducing Antioxidant Power (FRAP)

Ferric reducing antioxidant power (FRAP) is based on the ability of antioxidants to reduce ferric to ferrous ions (Table 23.2). Antioxidants present in the seminal plasma can reduce ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex to Fe^{2+}-TPTZ generating a blue-colored complex. Absorbance at 593 nm is measured over time. The comparison between the changes in absorbance from the beginning to the end of the test gives an estimate of the amount of iron reduced and can be correlated with the antioxidant capacity of the sample [42]. Trolox or ascorbic acid can be used as reference. A statistically significant positive correlation was observed between the TAC measured by FRAP and sperm parameters such as concentration, motility, and morphology ($p < 0.05$) [43].

2,2-Diphenyl-1-Picrylhydrazyl (DPPH)

This assay is based on the ability of antioxidants to reduce the stable organic nitrogen radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) to DPPH. Formation of DPPH upon absorption of hydrogen atom from an antioxidant results in the decrease of UV absorption at 515 nm that can be spectrophotometrically measured. This reaction is accompanied by a change in color from the initial deep purple color of DPPH• to a colorless product. Trolox is used as standard antioxidant [44].

Trolox Equivalent Antioxidant Capacity (TEAC)

This antioxidant assay is based on the principle that both aqueous and lipid antioxidants in the seminal plasma can reduce the stable radical 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) to ABTS. ABTS is prone to oxidation by peroxyl radicals or other oxidants present in the seminal plasma, forming a blue-green colored $ABTS^{\bullet+}$. The ability of antioxidants to reduce it causes a suppression of the absorbance at 750 nm and a simultaneous loss in color. The signal measured by spectrophotometry is proportional to the antioxidants' concentration. The capacity of seminal antioxidants to prevent ABTS oxidation is compared to that of standard trolox. Results are reported as micromoles of trolox equivalent [41].

Oxygen Radical Absorbance Capacity (ORAC)

This method measures the antioxidant scavenging activity against the peroxyl radical, induced by 2,2′-azobis-(2-amidino-propane) dihydrochloride (AAPH), at 37 °C. The peroxyl radical reacts with a fluorescent probe to form a non-fluorescent product which can be quantitated by a fluorometer. Antioxidant capacity is determined by a decreased rate and amount of product formed over time. Commonly used probes include fluorescein and H_2DCFDA, which are more stable and less reactive than the previously used B-phycoerythrin probe [44].

Enhanced Chemiluminescence

The enhanced chemiluminescence assay is the most commonly used method to measure the TAC in the seminal fluid (Table 23.2). This test was adapted from the total radical-trapping antioxidant parameter (TRAP) assay [44]. It allows the quantification of the ability of seminal antioxidants to block oxidation of specific reagents. A signal reagent (luminol plus p-iodophenol) is mixed with an oxidant source of peroxyl radicals (horseradish peroxidase (HRP)-linked immunoglobulin) to produce ROS, which in turn is mixed with a substrate, hydrogen peroxide (H_2O_2). The system

Table 23.2 Methods for the assessment of total antioxidant capacity (TAC)

Method	Type of sample	Principle	End-product determination	Advantages	Disadvantages
FRAP	Whole ejaculate Seminal plasma Spermatozoa	Measures the antioxidant potential through the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+})	Spectrophotometry	Measure the antioxidant capacity of all the antioxidants in a sample Simple Inexpensive Reproducible	Does not measure thiol antioxidants Low precision Time-dependent
DPPH	Seminal plasma	Measures the ability of antioxidants to reduce radical $DPPH^{\cdot}$ to DPPH	Spectrophotometry	Simple Rapid	Small linear reaction range Not a competitive reaction because DPPH is both radical probe and oxidant
TEAC	Seminal plasma	Measures the ability of antioxidants to reduce radical $ABTS^{\cdot+}$ to ABTS	Spectrophotometry	Simple Rapid Work in a wide pH range Determine both hydrophilic and lipophilic antioxidant capacities	Interference with peroxidases Do not differentiate water-soluble from lipid-soluble antioxidants
ORAC	Seminal plasma	Antioxidant reaction with peroxyl adicals, induced by AAPH	Fluorometry	High specificity Responds to several antioxidants Differentiates water- and lipid-soluble compounds Reliable	Time-consuming Temperature sensitive Requires specific equipment
Enhanced chemiluminescence	Whole ejaculate Seminal plasma	Antioxidant capacity to inhibit light production by luminol-derived radicals	Luminometry	Accurate High sensitivity High specificity Reproducible	Expensive equipment Time-consuming Limited half-lives of the probes Requires high sample volume

FRAP ferric reducing antioxidant power, *DPPH* 2,2-diphenyl-1-picrylhydrazyl, *TEAC* trolox equivalent antioxidant capacity, *ORAC* oxygen radical absorbance capacity, *AAPH* 2,2′-azobis-(2-amidino-propane) dihydrochloride

generates ROS at a known and steady rate, where the luminescence intensity remains almost constant for several minutes. This steady light emission is temporarily interrupted when an antioxidant is added to the system. The emission is subsequently restored once the ROS scavenging ability is depleted. The power of the seminal antioxidants to reduce the chemiluminescence of the signal reagent is compared with a standard. That standard is generally trolox, a water-soluble tocopherol analogue, and the results are reported as molar trolox equivalents [45]. In seminal plasma, the normal limit was established as TAC >2000 μmol of trolox [46].

23.2.3 ROS-TAC Score

Taking into account both sperm ROS levels and seminal TAC measurements by chemiluminescence, the parameter ROS-TAC score was suggested. This score reflects the overall level of oxidative stress (OS) by the balance between prooxidants and antioxidants in a sample. Based on ROS and TAC values from healthy donors, a series of equations to calculate the ROS-TAC of seminal sample have been proposed [47]. The ROS-TAC score showed a higher predictive value than ROS and TAC alone when it is used in discriminating fertile from infertile men. Infertile men have low ROS-TAC scores, and it was estimated that infertile men with higher ROS-TAC scores were more prone to initiate pregnancies than those who had low scores [47].

23.2.4 Oxidation-Reduction Potential (ORP)

23.2.4.1 What Is the Principle of ORP?

Oxidation-reduction potential (ORP) measures the redox potential of a semen sample or seminal plasma [48]. Redox reactions involve the transfer of electrons between two

Fig. 23.3 Representative image of a MiOXSYS system and sample loading into the disposable sensor trip

chemicals. The chemical that loses electrons is oxidized, while the one that accepts electrons is reduced. ORP provides an estimate of the proportion of oxidants and antioxidants present in the sample. This test can be performed by using the MiOXSYS system (Fig. 23.3). The sample is loaded through a disposable sensor strip, and the results are obtained in a few minutes. ORP measurement is based on Nernst equation E (ORP) = E_0 – RT/nF ln([Red]/[Ox]), where E_0 is the standard reduction potential relative to hydrogen electron, R universal gas constant, T absolute temperature in degrees Kelvin, n number of moles of exchanged electrons, F Faraday's constant, [Red] concentration of reduced species, and [Ox] concentration of oxidized species [49]. The results are given by the "static ORP" parameter (mV), which is a snapshot of the current OS in the sample. This value should be normalized to the sperm concentration in the semen sample (corrected ORP). This parameter gives an estimate of the antioxidant reserves expressed as mV/million sperm/mL [50].

23.2.4.2 What Is the Reference Value?

ORP values higher than the established reference values are indicative of OS. ORP values between 1.36 and1.42 mV/million sperm/mL showed the ability to distinguish fertile from infertile men [51–53]. ORP value of 2.59 mV/million sperm/mL is a good predictor of oligozoospermia [54]. It can be used for both fresh and frozen samples.

23.2.5 Clinical Value of ROS/TAC/ORP Measurement

Elevated ROS levels were detected in the semen of 25–40% of infertile men [47]. The implications of ROS on reproductive outcomes were further supported by the negative associations between increased ROS levels and traditional semen quality parameters [55, 56]. ROS assays may be complementary to conventional semen analysis in better assessing male fertility. This has a particular importance for patients with unexplained male infertility as a significant proportion of infertile men have normal semen parameters and are still infertile [57]. The possible value of measuring ROS may be related to the positive correlations found between high ROS levels and specific markers of male reproductive dysfunction, such as DNA fragmentation, leukocyte concentration, and the incidence of apoptotic or necrotic spermatozoa, which may not be detected by conventional semen analysis [57–59]. Elevated ROS were also associated with increased time to natural conception, impaired IVF pregnancy rates, and recurrent miscarriage [60, 61]. Measuring ROS levels in semen could help identify patients who may benefit from treatment that can potentially decrease OS such as oral antioxidant therapy, varicocelectomy, and lifestyle modifications. The response to the treatment can also be monitored by the ROS measurement. ROS may also play a role for ART. Elevated ROS levels in samples with poor quality may be exacerbated by manipulation and prolonged incubation during the procedures, which further impairs sperm function. Modifications of the media such as the application of antioxidants to minimize oxidative-induced sperm damage may provide its value in protecting sperm from ROS damage during ART.

Besides the increased ROS levels, some infertile men also demonstrate a 30–43% decrease in TAC relative to fertile controls and regardless of semen parameters [43, 62, 63]. In fact, decreased TAC was associated with impaired sperm function [40]. Accordingly, infertile men also have significantly lower ROS-TAC scores [47]. Many studies reported a positive correlation between standard sperm parameters such as concentration, motility and morphology, and TAC levels ($P < 0.05$) [43, 64]. Assessment of TAC in infertile men will provide a more complete picture in the assessment of OS as an etiology of male infertility. Analyzing both ROS and TAC in a semen sample can be an important complement to the conventional semen analysis. The findings from OS testing may alert the clinician to implement means to lower OS in addition to ART and other intervention. In fact, data from several studies indicate that men taking oral antioxidants have a four fold increase in pregnancy rates and an almost five fold increase in live birth rate when undergoing ART [65].

ORP has emerged as a simple test to evaluate OS, which could be easily performed in an office-based setting. The equipment is handy, the test uses very low amount of liquefied sample, the result is available in 2 min after loading the sample, and the measurements are stable up to 120 min [66]. It is also associated with a low intra- and interobserver variability [51]. Preliminary data show high sensitivity, specificity, and accuracy of ORP, which reflects its potential clinical application [48]. ORP levels were negatively correlated with sperm parameters [54]. It has the potential in distinguishing infertile from fertile men [51]. However, additional research is needed to gather solid scientific proofs for the implementation of OS assays in a clinical setting.

23.3 Evaluation of Sperm DNA Damage

23.3.1 What Is DNA Damage?

The maintenance of sperm DNA integrity is vital for fertilization and to transmit the genetic material to the offspring. Sperm chromatin undergoes extensive remodeling during spermatogenesis to protect sperm DNA. In this process, most of sperm DNA histones are replaced by protamines to attain a higher compaction. If these events do not occur properly and spermatozoa retain more histones than they should, sperm DNA becomes more susceptible to damage [67]. However, DNA damage can occur not only during spermatogenesis but also during epididymal transit or even when spermatozoa are collected for ART purposes [68].

DNA damage refers to defects in DNA structure, which include single or double DNA strand breaks, deletion or modification of DNA bases, interstrand or intrastrand DNA cross-linkage, and protamine mispackaging. Single- (ss) or double-stranded breaks (ds) are commonly termed DNA fragmentation. OS is one of the major extrinsic factors leading to sperm DNA damage, but it may also result from pathologies of the male reproductive system [69]. Every 25% increase in seminal ROS levels is associated with a 10% increase in sperm DNA fragmentation rate [62]. Additionally, abortive apoptosis, unresolved strand breaks during spermiogenesis, and defective maturation have also been implicated as potential intrinsic sources of DNA damage [69, 70].

23.3.2 How Can Sperm DNA Fragmentation Affect Sperm Quality?

DNA damage can affect both nuclear and mitochondrial DNA. OS-induced damage to nuclear DNA may accelerate germ cell apoptosis, leading to a decline in sperm production and consequently decreased sperm count in the ejaculate [16]. A positive correlation has been shown between sperm DNA fragmentation and abnormal morphology, which was associated with defects of the sperm tail [71]. Another study showed a higher incidence of aneuploidy in men with high sperm DNA fragmentation [72]. Alterations in mitochondrial sperm DNA may lead to impaired mitochondrial functionality, thus resulting in compromised motility, hyperactivation, and inability to fertilize. The ability of sperm DNA to repair damages is very limited, thus any alteration in DNA structure can lead to the dysfunction of sperm proteins. Although spermatozoa possess the base excision repair (BER) enzyme, they lack oxoguanine glycosylase (OGG1) that is essential for the repair process. This makes spermatozoa susceptible to DNA damages and dependent on the ability of the oocyte to repair any existent damage after fertilization [73]. However, when the DNA damage is extensive, the oocyte's repair capacity is not sufficient, and fragmentation can result in low rate of embryonic development and miscarriage.

23.3.3 Methods to Measure Sperm DNA Damage

Although the clinical utility of sperm DNA fragmentation has not been well-established, it has potential to become an important test for evaluation of male infertility. The available methods can be divided into assays that (i) measure DNA damage by incorporating DNA probes or modified nucleotides at the site of damage, (ii) measure the susceptibility of DNA to denaturation, and (iii) indirectly measure the level of chromatin compaction.

23.3.3.1 Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL)

This assay is based on the deoxynucleotide (dUTP) binding to the 3'-OH break-ends of single- and double-stranded DNA breaks. A template-independent DNA polymerase called terminal deoxynucleotidyl transferase (TdT) catalyzes the attachment of the dUTP [74]. Moreover, the dUTP are labeled with a fluorescent probe (e.g., fluorescein isothiocyanate), to allow the detection by fluorescent microscopy or flow cytometry. The fluorescent signal will be directly proportional to the number of strand breaks and will be reflected as a percentage of sperm DNA fragmentation. Recent advances in this area allowed an increase in the sensitivity of the test by improving the interaction of TdT with DNA strand breaks [75]. The cutoff values of sperm DNA fragmentation for TUNEL have not been clearly established [76]. A cutoff value of 17% was proposed to discriminate between infertile and fertile men with a high specificity (92%) and high predictive value (91%) [74]. TUNEL availability as a commercial kit is one of the advantages for the clinical implementation of this test (◘ Table 23.3). TUNEL will be described in more detail in ▶ Chap. 24.

23.3.3.2 Comet Assay

The Comet assay is a single-cell gel electrophoretic assay that quantifies broken strands of DNA in individual spermatozoon. First, sperm membranes are lysed and the DNA decondensed in a salt solution. This assay can only measure double-stranded DNA breaks in a neutral buffer, while in an alkaline environment, DNA is denatured so that both single- and double-stranded DNA breaks can be measured [77]. DNA breaks have different mobility when placed in an electrophoretic field depending on the relative size of the fragment. When the electric field is applied, all the broken strands of DNA (negative charge) will migrate toward the positively charged anode, forming a comet tail. Spermatozoa with more DNA breaks will show the most intense and largest comet tail [78]. DNA damage will be assessed using a fluorescence microscope or cytometer. The length of the comet tail and fluorescent intensity will be directly proportional to the degree of DNA fragmentation [79], and the result is presented as a percentage of DNA damage per spermatozoon. It was estimated that results from Comet assay are able to predict the fertilization rates with a 93.3% specificity.

Table 23.3 Methods for sperm DNA damage evaluation

Method	Principle	Advantages	Disadvantages
TUNEL	Directly measures single- and double-stranded DNA fragmentation	High sensitivity and specificity Available as commercial kits Measures a definite endpoint	Labor intensive Requires specific material and expensive equipment Not specific to oxidative damage Thresholds not standardized Results affected by preparation, fixation, and permeabilization
Comet	Quantifies the shape of the single cell nuclei after gel electrophoresis	Requires small number of spermatozoa (5000) High sensitivity Wide pH range Detects single- and double-stranded breaks, as well as altered bases	Labor intensive Requires expensive software High variability of protocols Intra- and interobserver variability Not specific for oxidative damage DNA damage can be overestimated
SCD	Indirect estimation of DNA fragmentation by quantification of the amount of nuclear dispersion (halo)	Differentiates sperm with fragmented and non-fragmented DNA Can use bright field or fluorescence microscopy	Interobserver subjectivity when categorizing the halos DNA damage can be overestimated
AO TB CMA3	Uses cytochemical dyes combined with bright field or fluorescent microscopy to assess sperm chromatin integrity	Simple Fast Inexpensive	Do not measure DNA fragmentation Intra- and interobserver variability Reference values not standardized Rapid fading of fluorescence
SCSA	Indirect analysis of sperm DNA damage by measuring the susceptibility to denaturation	Established clinical thresholds, robust, high sensitivity, requires few cells (10,000)	Involves complex calculations Do not give information about the amount of DNA damage in a single sperm

TUNEL terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling, *SCD* sperm chromatin dispersion, *AO* acridine orange, *TB* toluidine blue, *CMA3* chromomycin A3, *SCSA* sperm chromatin structure assay

A percentage of DNA fragmentation higher than 40% reflected a 9.5 times higher risk of poor fertilization [80].

23.3.3.3 Sperm Chromatin Dispersion (SCD)

Sperm chromatin dispersion (SCD) test is also known as the halo test due to its availability as a kit – Halosperm® kit [81]. This is a simple assay to estimate the amount of spermatozoa with DNA fragmentation. SCD allows the differentiation between spermatozoa with fragmented or non-fragmented DNA. Similar to comet assay, this test involves a single-cell agarose gel electrophoresis followed by denaturation with an acid or alkaline solution to remove nuclear proteins [82]. Slides can be visualized using bright field or fluorescence microscopy. Sperm showing halos of dispersed chromatin around a dense core are considered normal, while those with a small halo or no halo contain a fragmented DNA because the DNA loops do not diffuse [77].

23.3.3.4 Cytochemical Tests

Simple cytochemical tests can be used to assess sperm DNA integrity, including acridine orange (AO), toluidine blue (TB), and chromomycin A3 (CMA3) (Table 23.3).

AO is a membrane-permeable fluorescent dye that allows the assessment of sperm chromatin integrity. DNA is firstly exposed to an acid environment, which dissociates thiols from DNA thus increasing DNA susceptibility to denaturation. Consequently, AO competes for anionic binding sites by avoiding nonspecific aggregation. AO molecules bind to single-stranded DNA as an aggregate and are intercalated with normal double-stranded DNA. After preparing the slides, determination of DNA integrity is made under a fluorescence microscope. When exposed to wavelengths of 450–490 nm, sperm with normal DNA integrity will emit green fluorescence, while those with abnormal denatured/single-stranded DNA will emit red fluorescence. This allows the calculation of the percentage of spermatozoa with denatured DNA [83].

TB is a cationic dye that binds to negatively charged phosphate residues. It can be used for the identification of sperm chromatin abnormalities. When spermatozoa are stained with TB, those with a tightly bound DNA will have few TB binding sites and will be slightly stained. Spermatozoa with poor DNA integrity will expose phosphate residues, and they will stain dark (purple). Cells are analyzed under a bright light microscope, and the percentage of sperm with abnormal chromatin structure is calculated. A significant correlation was found between AO and TB tests ($R = 0.431$, $p < 0.001$) [84].

CMA3 is a specific GC-rich sequence dye that allows the indirect measurement of the amount of protamines in the

sperm nucleus. CMA3 interacts with DNA at the protamine binding sites. The intensity of CMA3 staining can be visualized under a fluorescent microscope and higher intensities indicate protamine deficiency or aberrant chromatin packing [85].

23.3.3.5 Sperm Chromatin Structure Assay (SCSA)

Sperm chromatin structure assay (SCSA) was developed based on the principle of the acridine orange test; however, it uses flow cytometry to analyze a large number of sperm. This assay estimates the percentage of sperm with higher susceptibility to DNA breaks rather than the amount of DNA damage in a single sperm. Staining by acridine orange is highly precise, reproducible, and comparable between fresh and frozen samples. SCSA allows the calculation of the DNA fragmentation index (DFI), which is the ratio between the percentage of spermatozoa showing red fluorescence (denaturated DNA) and the total fluorescence (red + green) [86]. High levels of DNA fragmentation (≥30% DFI) were associated with a higher risk for low blastocyst rates and failure to initiate an ongoing pregnancy [87]. Besides, it measures the percentage of sperm DNA stainability (% HDS) that is related to the nuclear histones retained in immature sperm. This test may provide important information for the physician before counseling couples to undergo IVF or ICSI. Men with HDS ≥15% demonstrated lower fertilization rates, indicating that ICSI may be recommended in those cases. Besides, men with DFI ≥30% were associated with a higher risk for pregnancy failure [87].

23.3.4 Clinical Value of Sperm DNA Damage Evaluation

Spermatozoa with a higher DNA integrity have a better chance to achieve fertilization and a successful pregnancy. Sperm DNA damage is often found in men with advanced paternal age, unhealthy diet, drug abuse, environmental pesticide exposure, tobacco use, varicocele, medical diseases, hyperthermia, air pollution, genital inflammation, and infectious diseases. Furthermore, sperm DNA damage is significantly increased in men with idiopathic and male factor infertility, which may be related to high levels of seminal OS [83]. However, men with apparently normal sperm parameters (e.g., motility and morphology) can also have high DNA fragmentation. In fact, DNA fragmentation has been pointed as one of the factors behind the commonly classified idiopathic infertility.

High rates of sperm DNA fragmentation were correlated with decreased semen quality [88]. Men with varicocele also present higher levels of sperm DNA fragmentation [89, 90], which may account for their reduced reproductive potential [91]. Sperm DNA fragmentation was also associated with longer time for natural conception, idiopathic infertility, recurrent intrauterine insemination (IUI) and in vitro fertilization (IVF) failure [92], and spontaneous miscarriage [93–95]. ROS-induced damages to mitochondrial and nuclear sperm DNA may also end up in increased birth defects and high offspring morbidity [96]. Currently, routine implementation of sperm DNA damage tests is not supported by guidelines. However, emerging clinical evidence indicates sperm DNA fragmentation as a useful biomarker for male infertility diagnosis and prediction of both natural and assisted reproduction outcomes. The clinical utility of sperm DNA fragmentation assays has been recently proposed in the following situations: (a) infertile men with a normal semen analysis as determined by conventional methods, (b) recurrent spontaneous abortion, and (c) to determine the most suitable ART [97].

23.4 Assessment of Sperm Fertilization Capacity

23.4.1 How Can We Measure the Sperm Fertilization Capacity?

For the spermatozoon to be able to fertilize, it needs to acquire fertilization potential during maturation through the epididymis. However, the ability to fertilize is only activated physiologically when in contact with the female gamete, or it can be induced in vitro using proper media. Sperm function testing also includes the evaluation of sperm mitochondrial function and fertilization events such as hyperactivation, capacitation, and acrosome reaction. Even an apparently normal spermatozoon with normal motility and morphology can be dysfunctional and not be able to fertilize. So, these tests may serve as a valuable tool to infer about the fertilizing potential of an individual before ART.

23.4.2 Methods to Assess Sperm Fertilizing Potential

Several methods have been used over the last decades to evaluate sperm interaction with the oocyte and binding to the zona pellucida. However, most of these tests are not commonly used currently due to the limited availability of human oocytes to conduct the experiments and also to the emergence of ICSI, which bypass the sperm-oocyte interaction. The binding of spermatozoa to the zona pellucida triggers the acrosome reaction of sperm and induces the release of acrosomal components (e.g., acrosin and hyaluronidase). So, the evaluation of acrosome reaction can have greater value to indicate if these mechanisms are occurring properly.

A proper mitochondrial function is vital for sperm function. Mitochondria are the power organelles of cells, essential to produce energy for sperm metabolism, membrane function, and motility. Loss of mitochondrial membrane potential is one of the triggers for cellular apoptosis [98]. Some of the tests used for acrosome reaction testing and evaluation of sperm mitochondrial function will be discussed. These types of tests may be useful when significant head morphological

abnormalities are found during routine semen analysis or in cases of idiopathic infertility, especially with repeated pregnancy failure with ART.

23.4.2.1 Acrosome Reaction Testing

Acrosomal integrity can be assessed by microscopy or flow cytometry using fluorescent lectins that selectively bind to either the outer membrane or acrosomal contents (Fig. 23.4) [99]. After inducing the acrosome reaction with the calcium ionophore A23187, spermatozoa are stained with a fluorescent probe. The fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) and fluorescein isothiocyanate-conjugated *Pisum sativum* (FITC-PSA) are the most commonly used probes [100]. As acrosomal loss can result from spontaneous acrosome reaction (<5%) or normal sperm death, this test is often used in conjunction with a test of cell viability, which can be done using the Hoechst staining. This test will give the percentage of acrosome-reacted viable spermatozoa [101]. Semen samples with 5–30% of reacted spermatozoa have a higher fertility potential [102].

Another test is based on the ability of spermatozoa to bind to hyaluronic acid. Only spermatozoa that are mature, viable, and with intact acrosomes will bind to hyaluronic acid through specific receptors [103]. This hyaluronic binding assay (HBA) has been used as a sperm selection technique for ICSI. The use of this procedure has been correlated with the selection of spermatozoa with high DNA integrity and low frequency of chromosomal aneuploidies [104].

23.4.2.2 Sperm Mitochondrial Activity

The fluorescent probe JC-1 can be used to distinguish between spermatozoa with poorly and highly functional mitochondria using flow cytometry. The accumulation of the JC-1 dye in sperm mitochondria depends on mitochondrial membrane potential [105]. JC-1 accumulates in the cytosol of healthy sperm as a monomer, whereas in the presence of high mitochondrial membrane potential, it accumulates as aggregates inside the mitochondria. Monomers will emit green fluorescence, while aggregates will emit red fluorescence. The JC-1 ratio aggregates/monomers give an estimation of mitochondrial functionality. When spermatozoa are dying, only JC-1 monomers (green fluorescence) will be present. This technique is simple and is the only method available for the evaluation of sperm mitochondrial function [106]. A recent study proposed that the evaluation of mitochondrial membrane potential in combination with sperm DNA fragmentation testing could be stronger predictors of natural conception relative to standard semen analysis [107].

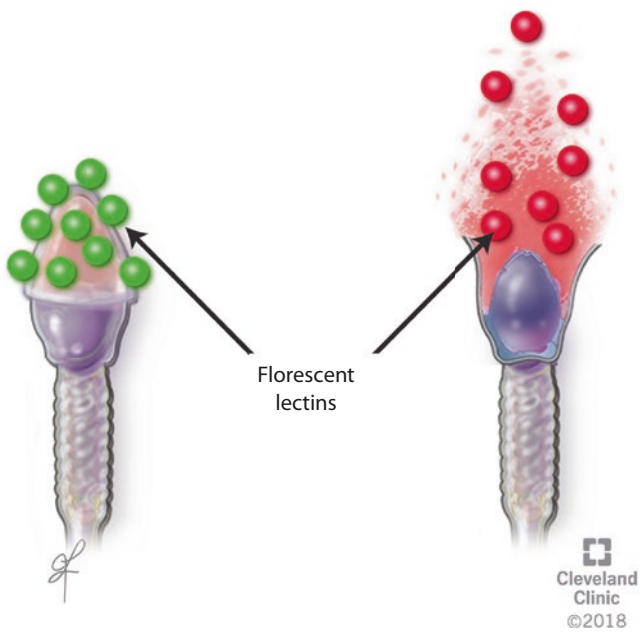


Fig. 23.4 Acrosome reaction testing by fluorescent lectins **a** or acrosomal enzymatic release **b**

23.5 Antisperm Antibodies

Among all the male infertility cases, about 10% are associated with the presence of antisperm antibodies (ASA) [108]. Observation of sperm agglutination during a routine semen analysis is usually an indication for ASA. The presence of ASA can affect sperm function by hindering sperm migration through the female reproductive tract and reducing the number of sperm at the fertilization site. Furthermore, ASA have been associated with the secretion of cytokines which impair sperm function and can eventually lead to cell death [109]. Only antibodies that bind to sperm membrane antigens or are found free in the seminal plasma are of functional significance. IgA and IgG classes are the most abundant, contrastingly to IgM [110]. Many tests have been developed for ASA diagnosis, but only the mixed antiglobulin reaction (MAR) test [111] or immunobead test (IBT) [112] is recommended by the WHO [113]. The MAR test is performed on a fresh semen sample. In this test, spermatozoa are mixed with latex particles which are coated with antibodies (anti-IgA or IgG). A monospecific anti-human-IgG or anti-human-IgA is added to the semen sample. The beads will bind to spermatozoa containing the respective antibodies on their surface. The antibody binding location (sperm head, mid-piece, tail, tail tip, or total sperm involvement) is determined. Agglutination can be seen under a light microscope (phase contrast) as mixed clumps of spermatozoa (Fig. 23.5a) [114].

In IBT, the seminal plasma is removed by centrifugation to isolate spermatozoa. Spermatozoa are mixed with beads that have been coated with IgG (or IgA) class-specific secondary antibodies. The sperm suspension is then observed microscopically for agglutination. The beads will bind to both motile and immotile spermatozoa with surface-bound antibodies (Fig. 23.5b), but only motile sperm should be recorded [115]. Antibodies are considered clinically significant when more than 50% of spermatozoa are coated, when spermatozoa are unable to penetrate the preovulatory human cervical mucus, or when spermatozoa demonstrate impaired fertilizing capacity [113]. Nevertheless, there is a controversy about the correlation of these tests with the reproductive outcome by ART [110].

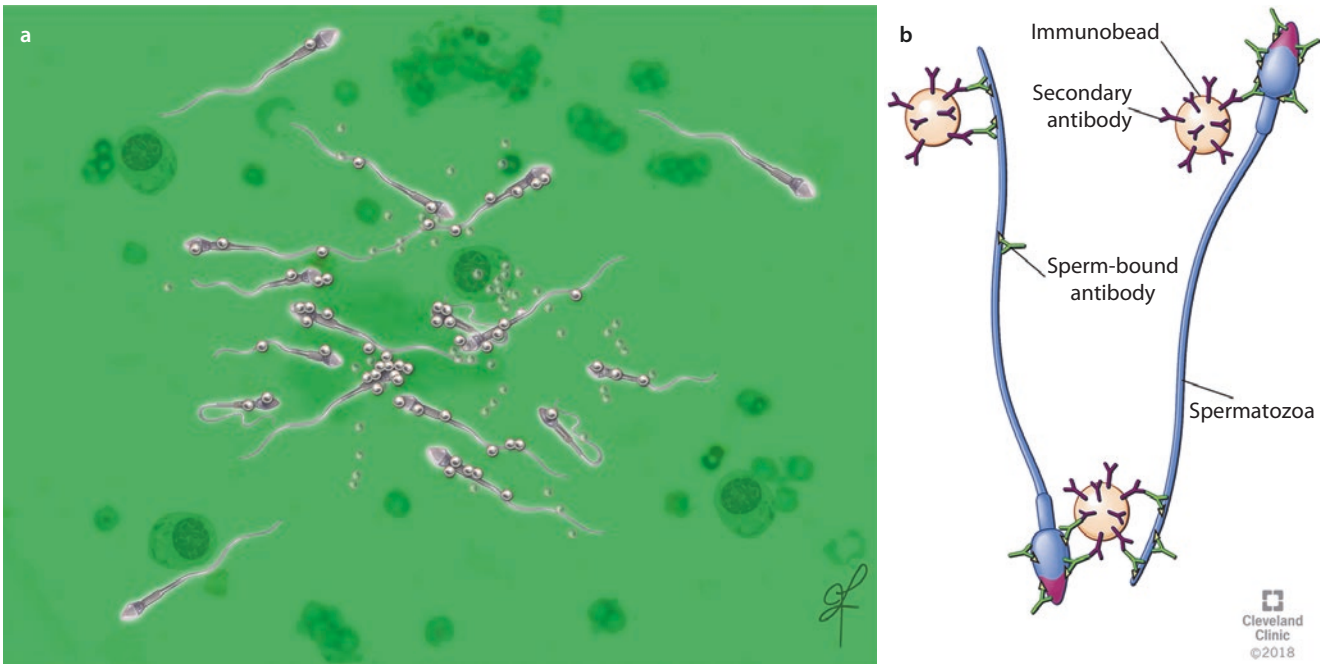


Fig. 23.5 Antisperm antibody testing: microscopic illustration of sperm and bead aggregates by mixed agglutination reaction (MAR) test **a** and immunobead binding test (IBT) principle **b**

Existing data do not support the widespread use of immune testing in clinical practice. Further research is required to determine the stimuli for ASA production and their class-specific effects on individual sperm proteins, which may lead to the development of new tests or therapies [116].

23.6 Future Perspectives

The continuous search for a method to accurately assess fertility potential stimulated the in-depth investigation of the quality and function of the gametes. New approaches are constantly under appraisal. Digital holography microscopy applied to both morphological and motility characterizations of sperm cells has been proposed. The idea is to use quantitative three-dimensional information (e.g., volume, profiles along particular directions) to better underline the differences between normal and abnormal sperm morphology [117]. Besides, advances in biomolecular techniques pointed out promising targets for the development of novel laboratory tests. In particular, the study of semen genomics, transcriptomics, proteomics, and metabolomics is providing new insights into the biochemical basis of defective semen quality. This information can be used to enhance the understanding of the causes of male infertility and in the development of new tools for diagnostic and therapeutic purposes.

23.7 Concluding Remarks

Over the last decades, the necessity for sperm functional testing to overcome the limitations of routine semen analysis has become clear. Although the current available tests can provide important information in counseling and management of infertile couples, the lack of standardization and refinement of the assays represents the major obstacle to wide clinical implementation. Sperm function tests can be a valuable complement to conventional semen analysis for the evaluation of fertility status and to predict the reproductive outcomes either by natural conception or ART. They may also contribute to the identification of causes for idiopathic and unexplained infertility.

Review Questions (MCQ Format)

1. What are the limitations of conventional semen analysis?
   (a) Poor correlation with reproductive outcomes
   (b) Lack of standardization between laboratories
   (c) Inherent subjectivity that lead to intra- and interobserver variability
   (d) All of the above

2. Which of the following parameters are evaluated by sperm function tests?
   (a) Production of reactive oxygen species (ROS)
   (b) Sperm total antioxidant capacity (TAC)
   (c) Sperm DNA integrity
   (d) All of the above

3. Which of the following points support the clinical use of sperm functional laboratory tests?
   (a) They are less subjective than routine semen analysis

(b) They may contribute to identify causes for idiopathic infertility
(c) A single parameter of sperm function analysis can determine a subject's fertility status
(d) All of the above

4. Which of the following sperm function tests have defined reference values by the WHO 5th edition?
(a) Antisperm antibodies by MAR or IBT tests
(b) Sperm DNA damage by TUNEL
(c) ROS levels by chemiluminescence
(d) All of the above

5. Which of the following statements about the current status of sperm functional testing are correct?
(a) Sperm function tests will replace conventional semen analysis
(b) There is sufficient evidence to support the routine clinical implementation of sperm function tests
(c) More studies are needed to establish reference values for sperm function tests before its clinical use
(d) None of the above

Answers

1. (d)
2. (d)
3. (b)
4. (a)
5. (c)

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Assessment of Sperm Chromatin Damage by TUNEL Method Using Benchtop Flow Cytometer

Ana D. Martins, Rakesh Sharma, and Ashok Agarwal

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Learning Objectives

- Sperm DNA chromatin packaging of the nucleus occurs during spermiogenesis. The chromatin becomes highly condensed and protamines replace most of the histones. With this high rate of compaction, the DNA is more stable, compact, and resistant to damage. Deficiency of protamines in sperm is related with high risk of DNA damage.
- The major causes of abnormalities in spermatozoa are errors in meiotic recombination, abortive apoptosis, or excess of oxidative stress resulting from exposure to toxic agents or an infection.
- The techniques to evaluate sperm DNA fragmentation are TUNEL assay, comet assay, sperm chromatin dispersion (SCD) test, and sperm chromatin structure assay (SCSA).
- Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay cutoff of 17% with 92% specificity can differentiate infertile men with DNA damage from healthy men.
- The clinical value of including sperm DNA fragmentation is still under discussion; however, in recent years a negative relation between sperm DNA fragmentation and pregnancy rates has been reported.

24.1 Introduction

Spermatozoa are unique cells that are designed to transfer the intact haploid genome to the oocyte. Once spermatogenesis is completed, spermatozoa are on its journey with the final goal of fertilizing the oocyte. To survive this journey and accomplish this goal, spermatozoa experience many significant adaptations in cell organization and function that are different from the somatic cells. In somatic cells, epigenetic modulations and modifications in histone tails control the chromatin structure and transcription of DNA. Spermatozoa are transcriptionally inert and DNA histones are replaced by protamines [1, 2]. Extrinsic factors such as storage temperatures, handling conditions, infections, and post-testicular oxidative stress or gonadotoxic effects of a treatment can cause injuries to spermatozoa DNA. However, sperm DNA is more susceptible if chromatin packing is incomplete during spermiogenesis at the time of protamine replacement. Intrinsic sources of sperm DNA damage include abortive apoptosis, errors in recombination, or oxidative stress.

Assisted-reproductive techniques (ART) have made successful pregnancies possible even when semen parameters are below the reference values established by WHO guidelines [3]. Basic semen analysis remains the first option for assessment of male fertility [4]; however, this analysis fails to predict the fecundity [4]. Consequently, men with normal semen parameters can still be infertile and are considered to present with unexplained or idiopathic fertility [5–8]. Many studies report an association between the increase in DNA fragmentation and the prevalence of infertility [9–12]. Increase in DNA fragmentation is related with an increase in the time to achieve pregnancy [9], the miscarriage rate [13–15], and decrease in the success rate of in vivo fertilization [11, 16]. After fertilization, the oocyte and the embryo have a limited capacity to repair sperm DNA damage [17, 18]. All together, these studies highlight the importance of the evaluation of DNA fragmentation in the treatment of male infertility. The assessment of DNA damage dates back to 1970, when Ringertz and collaborators showed DNA denaturation with acridine orange by microfluorimetry [19]. Later on in 1980, with the progress of advanced molecular technologies and with the discovery of flow cytometry, the sperm chromatin structure assay (SCSA) was developed [20]. In the next decade, the progress of the single cell electrophoresis allowed the development of the comet assay [21]. Also, during these years, the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was developed to detect DNA fragmentation in human spermatozoa [22]. This technique will be the focus of this chapter. Recently the sperm chromatin dispersion (SCD) technique was developed for measuring DNA damage [23].

24.2 Sperm Chromatin Damage

24.2.1 Sperm Chromatin

In the somatic cell, chromatin is condensed and packed with histones [24]. Through post-translational modifications, histones are responsible for regulating DNA compaction [25] and modulating gene expression by controlling the access of transcription factors to the DNA [25]. The DNA from somatic cells can be packaged in two types: (1) in a nucleosome, a protein formed by an octamer of histones wraps DNA twice, and (2) nucleosomes packaged in solenoids [26–28]. In sperm DNA, the packaging of chromatin is different [26]; throughout spermiogenesis, in order for the sperm nucleus to become highly compacted, majority of the histones are replaced by protamines [29]. Compared to histones, protamines are simpler and smaller [2]. They also have arginine that allows a stronger binding to the DNA. In the final step of sperm epididymal maturation, the protamines undergo cross-linking by disulfide bond formation in the presence of cysteine residues [27]. During the transit through the epididymis, the chromatin volume is compacted six fold [1]. This compaction allows the DNA to be highly stable and resistant to damage. Sperm DNA with protamine deficiency in infertile men is associated with high risk of DNA damage [30–32].

24.2.2 Types of Damage to Spermatozoa

The origin of the human sperm chromatin damage is multifactorial [33]. Many factors are responsible for the disturbances in the highly sensitive biochemical events occurring during spermatogenesis, such as environmental stress [34] and chromosomal abnormalities [35].

24.2.2.1 DNA Fragmentation

DNA fragmentation can be attributed to many factors [36]. These factors can be intrinsic, such as apoptosis and oxidative stress [8, 37], defects in protamination [38], inefficient chromatin remodeling, and impairment of spermatogenesis [39, 40]. On the other hand, external factors such as lifestyle (diet, drug consumption, tobacco) [41–44], advancing age [45–47], environmental exposure (pesticides and air pollution) [48, 49], and medical history (cancer, varicocele, inflammation) [50–52], among others, can also result in sperm DNA fragmentation. Post-testicular damage is one of the most common causes of DNA fragmentation. The damage can occur after spermiation, during transit of the spermatozoa from the seminiferous tubules to the epididymis [53]. Studies show a higher DNA fragmentation in epididymal and ejaculated spermatozoa compared with testicular spermatozoa [53, 54].

24.2.2.2 Abnormal Chromatin Compaction

One of the most important steps in chromatin remodeling during spermiogenesis is the replacement of histones by protamines. This remodeling is assisted by hyperacetylation of the histones and by DNA topoisomerase II which is responsible for the formation of temporary nicks to release the tension that results from supercoiling [55, 56]. Topoisomerase II also repairs the temporary nicks after spermiogenesis and ejaculation. When this process fails, spermatozoa with DNA fragmentation are seen in the ejaculate [57].

24.2.2.3 Chromosomal Aberrations

Chromosomal aberrations affect 2–14% of the infertile men. Both numerical and structural aberrations can result in pregnancy loss, perinatal death, and congenital malformations [58]. Aneuploidies of X or Y chromosome, trisomy of chromosomes 13, 18, and 21, are the most common [59]. Normally, sex chromosome aneuploidies have paternal origin and are attributed to errors during spermatogenesis [60]. The homologous chromosomes are separated during the first and second meiotic division; if an error occurs at this time, this may lead to aneuploid gametes affecting autosomes or sex chromosomes. In the male, aneuploidies are hypothesized to occur by the following mechanisms: (1) during anaphase, chromosomes lag near the equator resulting in the posterior loss of the chromosome, (2) lack of separation of the chromatid pairs during mitosis or meiosis II, and (3) nonseparation of homologous chromosomes during meiosis I [59, 61].

24.2.3 Causes of Sperm Chromatin Damage

Over the years, the number of studies comparing DNA fragmentation with male fertility has increased. A high percentage of sperm DNA fragmentation is seen in men with idiopathic infertility. This may be a result of an increase in the availability of sperm DNA fragmentation assays and its relationship with fertility status. DNA is more vulnerable to oxidative stress and damage associated with the production of reactive oxygen species (ROS) in the absence of repair mechanisms and the transcriptional and translational inactivity in spermatozoa [51, 62, 63]. Abnormalities in spermatozoa can be due to errors in meiotic recombination, abortive apoptosis resulting from exposure to toxic agents, or excess of oxidative stress.

24.2.3.1 Meiotic Recombination

During pachytene stage, meiotic recombination is a crucial event for integrity of the genome and fertility. The number and the localization of this event is highly controlled; however, errors can occur resulting in aneuploid spermatozoa [64]. Compared with fertile men, nonobstructive azoospermic men present either without any meiotic cells or a reduced population of these cells caused by a complete or partial block at the zygotene stage [65]. The absence or reduction in meiotic recombination can result in sperm aneuploidy and spermatogenic arrest, resulting in a reduction of total sperm count and consequently infertility [65, 66].

24.2.3.2 Abortive Apoptosis

With the process of spermatogenesis, germ cells lose their apoptotic capacity, as they are transcriptionally and translationally silent. Sertoli cells are responsible for screening of the germ cells and initiating apoptosis, where up to 60% of all germ cell in meiosis I marked with the *Fas* type marker [67] are phagocytized and removed by the Sertoli cells [62, 68]. Infertile men present a higher incidence of *Fas* positivity [69]. Burrello and colleagues suggested that during spermiogenesis, a dissociation between sperm remodeling and genomic quality occurs, and this can be the reason why morphologically normal cells have aneuploidies or damaged nucleus [70]. When this process fails, some spermatocytes escape elimination and result in release of spermatozoa with increased DNA damage [63], and some of these spermatozoa are able to complete fertilization, although the probability of a live birth is very low [71].

24.2.3.3 Infections

Epididymis and secondary sexual glands are responsible for the presence of leukocytes in the ejaculate. These leukocytes play an important role in removal of abnormal spermatozoa [72] and immune surveillance [73, 74]. Leukocytospermia is indicative of infection and/or inflammation of the male accessory sex glands. Leukocytes are known high ROS producers and consequently result in DNA fragmentation and modifications in DNA bases. However, the effects of leukocytes are not only dependent on the number but also on the activation state [75, 76]. In a physiological state, ROS produced by leukocytes are scavenged by the antioxidants present in the seminal plasma. However, when this balance between ROS production and its effective removal by antioxidants is disturbed, it will result in oxidative stress and sperm DNA damage.

24.2.3.4 Exposure to Toxic Substances

Exposure to toxic substances is particularly harmful to male germ cells, resulting in DNA damage. Many studies report increased DNA fragmentation in men exposed to toxic substances as a result of their occupation, such as coke oven, waste incineration, and factory workers exposed to chemicals [77–79]. Cancer therapies have demonstrated adverse effects on sperm DNA integrity [80, 81]. Radiotherapy has been associated with higher DNA fragmentation after the treatment [82]. Similarly, the lifestyle factors such as men who smoke have higher DNA damage when compared with nonsmokers [83]. Consumption of alcohol and the use of illicit drugs were reported to increase sperm DNA damage [83].

24.2.3.5 Oxidative Stress

Oxidative stress is a subject of discussion in different fields, and male fertility is not an exception. Unlike the other cells, mature spermatozoa lack major DNA repair machinery. Spermatozoa from the testis seem to have low DNA fragmentation when compared to ejaculated spermatozoa [84]. Compared to fertile men, higher levels of oxidative stress are seen in infertile men [85]. Studies show an increase in DNA fragmentation and damage when sperm were exposed to hydrogen peroxide [86]. Sperm DNA fragmentation induced by ROS results in strand breaks, base modification, as well as gene mutations such as polymorphism [87].

24.2.4 Evaluation of Sperm Chromatin Damage

Sperm DNA fragmentation can be measured by a variety of assays that include the direct assays such as TUNEL, comet, and in situ nick translation test. These assays measure the amount of DNA fragmentation using probes and dyes. Indirect assays such as SCSA measure the susceptibility of DNA denaturation to acid denaturation, and SCD test measures the extent of halos formed based on the DNA integrity. The TUNEL assay, the SCSA, and SCD assay are the more common tests utilized for measuring DNA damage.

24.2.4.1 Sperm Chromatin Structure Assay

SCSA was developed during the 1980s. It is one of the most utilized assays to characterize male infertility with sperm DNA damage and chromatin abnormalities. The principle of the SCSA is based on the detection of DNA fragmentation using a flow cytometer, and large numbers of cells can be evaluated [88]. In this assay, following acid denaturation, sperm are stained with acridine orange—a fluorescent cationic dye [89]. The extent of acridine orange staining is determined by measuring the shift in metachromatic staining from green fluorescence to red fluorescence [84]. DNA fragmentation index (DFI) is the most important parameter. DFI is described as a percentage ratio of red fluorescent to green + red fluorescent, and it represents the population of cells with detectable denatured single-stranded DNA. One of the advantages of this assay is it is fast and robust, with a standardized protocol and small interlaboratory variation [88]. However, the need for a flow cytometer is a disadvantage to andrology labs due to the equipment cost. Values of DFI of 20–25% are related with infertility problems [11, 90]. When the DFI > 26% the fertilization can occur, however, most of the times this results in repeat pregnancy loss [91, 92]. In natural conception and intrauterine insemination (IUI), a DFI > 30% was associated with a negative pregnancy outcome [93, 94]. The cutoff values predicting ART outcomes are inversely proportional to DFI; when the DFI increases, the success of ART decreases [11, 90, 93, 94].

24.2.4.2 Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling Assay

The TUNEL assay principle is based on the addition of deoxyribonucleotides to 3′hydroxyl (OH)—single- and double-stranded DNA by a template-independent DNA polymerase called terminal deoxynucleotidyl transferase (TdT; ◘ Fig. 24.1). The terminal deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) is the substrate added by the TdT enzyme to the free 3′-OH breaks-end of DNA. This assay uses fluorescent nucleotides in the detection of "nicks" or 3′OH free ends of DNA. The samples can be evaluated by flow cytometry (◘ Fig. 24.1) or by fluorescence microscopy. However, the standardization between laboratories is still lacking, which makes the comparison and establishing a uniform threshold challenging [95]. A modified TUNEL protocol using a benchtop flow cytometer can accurately measure a large number of samples [96]. This assay is rapidly gaining popularity among other tests of DNA fragmentation. The assay has high sensitivity and specificity [97]. A cutoff of 17% with >95% specificity can distinguish infertile men with DNA damage from healthy men [96].

In Situ Nick Translation

In this assay the incorporation of biotinylated dUTP on single-stranded DNA breaks by the template-dependent enzyme DNA polymerase I [98] is measured. The test allows the identification of sperm that contain low but varying levels of endogenous DNA damage. It is less sensitive than other tests of DNA fragmentation. There is a lack of correlation of this test with fertilization rates during in vivo studies, limiting the clinical value of the assay [99].

24.2.4.3 Comet Assay

The comet assay measures the amount of DNA damage per spermatozoon using the single cell gel electrophoresis assay [100]. It is a simple and affordable assay to measure sperm DNA damage. The comet assay not only detects single- and double-strand breaks but can also identify altered DNA bases. The DNA fragments coming out of the sperm head resemble a "comet" tail and hence the name of the assay.

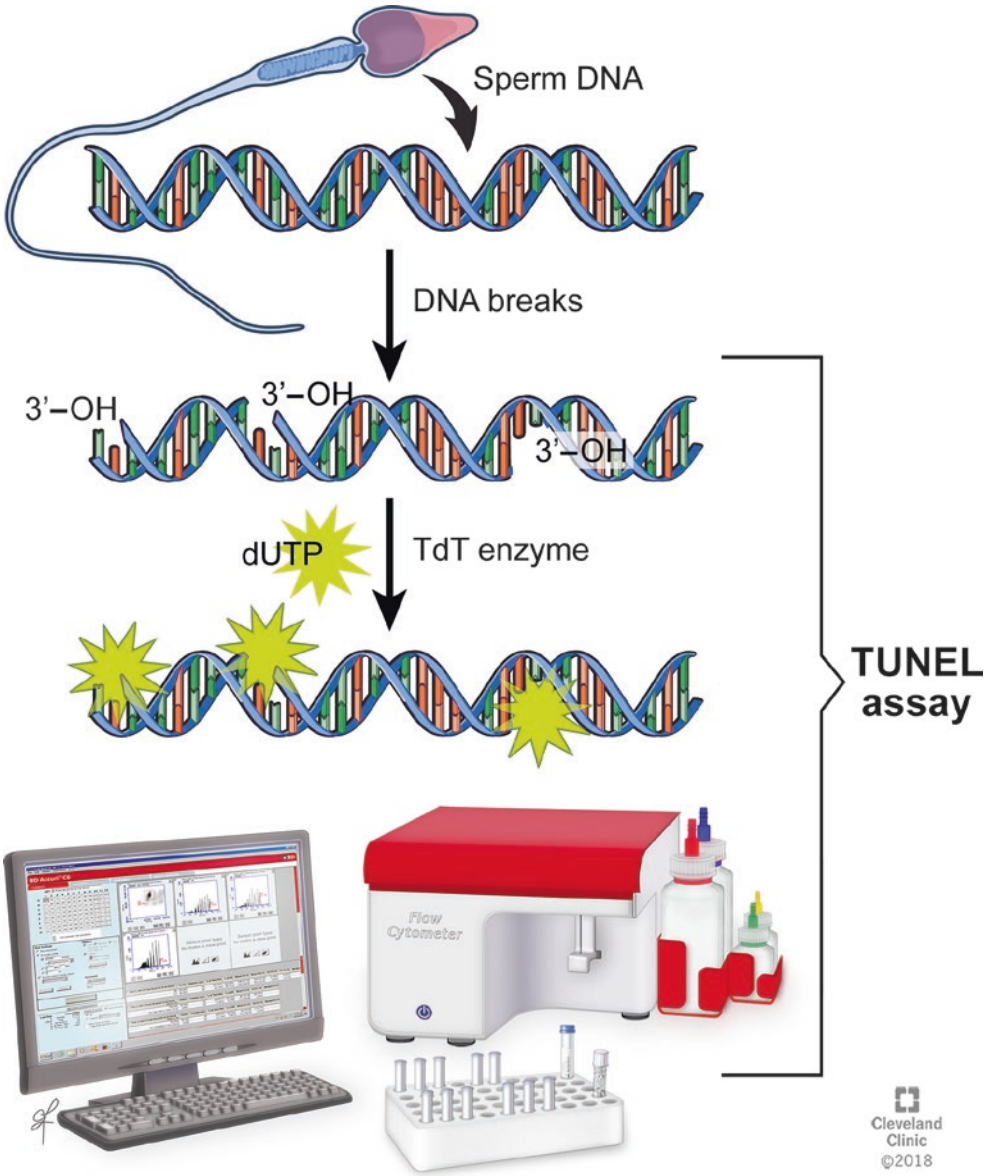

Fig. 24.1 Schematic of the DNA staining by the TUNEL assay

The assay principle is sperm nuclear DNA in an electric field can be separated based on the charge and fragment size that can be stained with a fluorescent dye. The results are qualitative and can be analyzed and graded using a fluorescence microscope, or quantitative and analyzed using an analysis software package [71]. Different types of DNA damage can be analyzed in a single cell and it requires highly specialized personnel and is time consuming [88]. The comet assay can be used to test samples from men with low sperm count. Results of the comet assay correlate with the results from the TUNEL assay [21].

24.2.4.4 Sperm Chromatin Dispersion Test

The SCD test is used to detect DNA fragmentation in spermatozoa using a kit called Halosperm® [101]. The principle of this assay is based on the fact that spermatozoa with fragmented DNA do not present the characteristic halo that is observed in spermatozoa with non-fragmented DNA following acid denaturation and removal of nuclear proteins [88]. In this assay, spermatozoa are immersed in an agarose matrix slide, and the nuclear proteins are removed by the denaturing solution exposing the damaged DNA. After lysis, spermatozoa show the characteristic loops around the sperm nucleus that correspond to spermatozoa with intact DNA; spermatozoa with damaged DNA do not exhibit any loop [88]. This assay can be done using bright-field microscopy with eosin and azure B staining or by fluorescence microscopy using DNA fluorescent probes [88]. It is simple, inexpensive, fast, and highly reproducible and does not require any complex instrumentation. However, this technique has some disadvantages, such as the interobserver variation. The halo boundary cannot be accurately differentiated from the background, the halos are not in the same plane, and some of them can be misread by an analysis software; sometimes the tails are not preserved and the contamination by other cells can be a challenge [87].

24.2.5 Effects of Sperm Chromatin Damage on Pregnancy Outcomes

24.2.5.1 Spontaneous Pregnancy

Many studies have reported that sperm from men with infertility may contain numerical or structural chromosomal defects, and karyotyping is the standard test to evaluate the chromosomal content [102]. Measuring sperm DNA fragmentation is important in the prognosis of achieving a natural pregnancy in couples. TUNEL and SCSA are used for testing the DNA fragmentation in infertile couples that present with recurrent pregnancy loss [8, 9]. A study showed low DNA damage levels, assessed by TUNEL in proven fertile men (11.9 ± 6.8%) when compared with men of unproven fertility (29.5 ± 18.7%) [103]. Another study comparing the sperm DNA fragmentation, assessed by TUNEL, of proven fertile men vs. patients where the partner had recurrent pregnancy loss showed significantly higher DNA damage in patients when compared to proven fertile men [104]. In fact, a higher probability of a successful pregnancy is reported, when men present with a low sperm DNA fragmentation [105]. Zidi-Jrah and colleagues reported a higher percentage of TUNEL-positive (cutoff >20%) sperm in a group with repeated pregnancy loss when compared to proven fertile men [106].

24.2.5.2 Assisted Reproductive Techniques

The effect of sperm DNA fragmentation in the success of pregnancy is dependent on the ART used. In case of in vitro fertilization (IVF), Henkel and colleagues showed a significant correlation between the low pregnancy rates and percentage of TUNEL-positive sperm. The pregnancy rate was 18.7% for TUNEL-positive spermatozoa, while in TUNEL-negative spermatozoa the pregnancy rate was 34.7% [107]. Another study reported a high percentage of DNA damage assessed by TUNEL and lower pregnancy rates in men undergoing IVF [108]. In case of IUI, no pregnancies were reported when the sperm had >12% of DNA fragmentation assessed by TUNEL [109]. Also in IUI cycles, Bungum and colleagues reported significantly lower rates of biochemical pregnancy, clinical pregnancy, and delivery rates in women inseminated with sperm with DFI higher than 30% [110]. Concerning the ART success rates when sperm DNA fragmentation was evaluated, studies demonstrate lower pregnancy rates when the sperm DNA fragmentation is high, but this is not true for intracytoplasmic sperm injection (ICSI) [111]. However, several studies demonstrate an association between the sperm DNA fragmentation and the success rate of ICSI. Host and colleagues reported a negative correlation between DNA fragmentation and fertilization rates where spermatozoa were used for IVF, but not for ICSI [112]. Henkel and colleagues did not find any correlation between TUNEL-positive spermatozoa (>36.5% DNA fragmentation) and fertilization rate using ICSI [107]. Huang and colleagues reported a negative correlation between sperm DNA fragmentation assessed by TUNEL assay and fertilization rates in cases of IVF and ICSI; however, the ICSI group seemed to be less affected [113]. In case of ICSI, Boroni and colleagues demonstrated an increase in pregnancy loss when sperm DNA fragmentation was high [114].

24.3 TUNEL Assay

24.3.1 Principles of TUNEL Assay

Sperm DNA fragmentation (SDF) is a result of activation of the endonucleases which break high-order sperm chromatin into smaller DNA fragments of ~50 kb [57]. DNA strand breaks are labeled by the fluorescein isothiocynate deoxyuridine triphosphate (FITC-dUTP) stain in the presence of TdT. TdT helps transfer the deoxynucleotides to the 3-hydroxyl (3-OH) end of the single- and double-strand breaks (Fig. 24.1). The intensity of labeling is proportional to the number of DNA strand break sites. SDF can be assessed with the benchtop BD Accuri C6 flow cytometer using the APO-Direct kit (BD Pharmingen, Cat. No. 556381) [115]. The kit consists of wash buffer, rinse buffer, reaction buffer, FITC-dUTP, TdT enzyme, and propidium iodide (PI)/RNase staining buffer. In addition, the kit also contains the "Negative control" and the "Positive control" (Fig. 24.2).

24.3.2 Why Should TUNEL Assay Be Used?

The basic semen analysis is the initial approach in the diagnosis of male reproductive status [3, 4]; nevertheless, sometimes this approach fails in the complete understanding of fertility potential in men seeking treatment [4, 116]. Over the years, the attention shifted to molecular structure of spermatozoa [28, 88]. The sperm DNA integrity is related with fertilization and consequent embryo development [28, 88, 117]. Although testing of sperm DNA damage is not recommended routinely for clinical use by the American Society of Reproductive Medicine [118], the importance of testing sperm chromatin fragmentation is recognized and approved by the American Urological Association and European Association of Urology guidelines on male infertility [88]. Based on the increased availability of this test, there is an increase in the number of studies reporting the correlation between sperm DNA fragmentation and successful pregnancies [12, 35, 84, 106, 119–121]. Although TUNEL is considered the gold standard for the evaluation of sperm DNA fragmentation [8], it lacks standardization, and the correlation with clinical outcome is still not clear [88, 94].

24.3.3 Advantages/Disadvantages of TUNEL Assay

TUNEL assay has the pros and cons for its use. One of the advantages of this assay using the flow cytometer is that it

Fig. 24.2 Components of the staining reagents: reaction buffer, TdT enzyme, and FITC vials

can be performed in a sample with low concentration of spermatozoa; it is rapid, robust, and highly sensitive; it is an indicator of apoptosis and can be correlated with semen parameter and consequently fertility; it has minimal interobserver variability; and there are commercial kits available to perform the analysis [28, 103, 122, 123]. On the other hand, the disadvantages of this assay are: it requires a flow cytometer and the threshold is not standardized between labs.

24.3.4 Methodology

24.3.4.1 Sample Preparation for TUNEL Assay

After complete liquefaction, aliquots containing 2.5 × 10^6/mL of sperm are prepared in duplicate, and tubes are labeled as "Test sample," "Negative control," and "Positive control." The "Test sample" and the "Negative control" tubes are centrifuged at 300 × *g* for 7 min; the supernatant is removed and replaced with 1 mL of phosphate-buffered saline (PBS).

Preparation of the Internal "Positive Control" Sample

In the tubes labeled as sperm "Positive control," a working hydrogen peroxide solution is prepared from stock hydrogen peroxide (37%) with PBS (1:15 dilution). One milliliter of the working solution is added to the sperm cells and the tubes are incubated at 50 °C for 60 min. After incubation, the tubes are centrifuged for 7 min at 300 × *g*; the supernatant is aspirated with a transfer pipette and resuspended in 1 mL of PBS 1×. After centrifugation at 300 × *g* for 7 min, the supernatant is removed and replaced with 1 mL of PBS.

The "Test sample," "Negative control," and "Positive control" tubes are centrifuged for 7 min at 300 × *g*. The supernatant is removed and replaced with 1 mL of 3.7% paraformaldehyde for "Fixation." After incubating the samples at room temperature for 15 min and centrifuging at 300 × *g* for 4 min, the paraformaldehyde is carefully aspirated and replaced with 1 mL of ice-cold ethanol (70%).

24.3.4.2 TUNEL Staining Protocol with the APO-Direct Kit

Staining Protocol

The "Negative controls" and "Positive controls" are provided as part of kit components (Fig. 24.2). These are cell lines and not sperm cells. The "Negative controls" and "Positive controls" are vortexed, and 2 mL suspensions of the well-mixed samples are aliquoted into the tubes. "Internal controls"—both positive and negative (two of each)—are also included with each run. The internal "Positive samples" are spermatozoa samples with known DNA fragmentation. The "kit control" samples, "Test samples," and the internal "Positive control" and "Negative control" samples are centrifuged for 7 min at 300 × *g*. After removing the 70% ethanol,

1 mL of the "wash buffer" is added, and the tubes are quickly vortexed and centrifuged at 300 × *g* for 7 min. The supernatant is removed, and this step is repeated. The tubes are numbered consecutively beginning with the kit "Negative control," "Positive control," "Test samples," and "Internal 'Negative' and 'Positive controls.'"

Staining for TUNEL Assay

To prepare the stain for TUNEL assay, the "reaction buffer" vial stored at 4 °C, the "TdT" vial, and "FITC-dUTP" vials stored at −20°C are placed at room temperature for 20 min. The staining solution is prepared by adding the reagents in the sequence shown in ◘ Table 24.1.

All the steps for preparation of the stain must be carried out in the dark. Negative internal controls are prepared by omitting "TdT" from the stain. After adding 50 µL of the "Staining solution" to each tube, the tubes are vortexed and incubated for 60 min at 37 °C. After incubation, 1 mL of "rinse buffer" (red cap) is added to each tube, and the tubes are centrifuged for 7 min at 300 × *g*. The supernatant is carefully removed, and the same step is repeated. Following centrifugation for 7 min at 300 × *g*, the supernatant is aspirated and discarded. The pellet is resuspended in 0.5 mL of PI/RNase buffer, and the tubes are incubated for 30 min at room temperature before running the samples on the flow cytometer.

24.3.4.3 General Setup of the Benchtop Cytometer

After double-clicking the "BD Accuri C6 software" icon on desktop, the software is opened; the reagent bottles are examined to ensure that the fluid levels are fine. The "waste" bottle should be empty, and the "sheath," "cleaner," and "decontamination" bottles must be full. The flow cytometer is turned on by firmly pressing on the power button. The "Traffic light" will turn yellow indicating that the peristaltic pump is working. Allow 5 min for the fluidic line to get flushed with the sheath fluid. Once the cytometer software light turns green, it indicates that the C6 Accuri is connected and ready. The tubing is flushed to remove any bubbles from the cytometer system. A 0.22 µm deionized (DI) water tube is placed on the sheath injection port (SIP) holder. The selection criteria for running the sample are "Run with limits" and "Fluidics" speed as "Fast." Once this is selected, click the "Run" button. At the end of the "flush cycle," the SIP tube is left on the SIP holder.

◘ Table 24.1 Preparation of stain for a single or multiple assay

Staining solution	1 Assay (µL)	6 Assays (µL)	12 Assays (µL)
Reaction buffer	10.00	60.00	120.00
TdT enzyme	0.75	4.50	9.00
FITC-dUTP	8.00	48.00	96.00
Distilled H_2O	32.25	193.5	387.00
Final volume	*51.00*	*306.00*	*612.00*

24.3.4.4 Instrument Quality Control

The quality control for FL1, FL2, and FL3 channels is performed and validated with 8-peak beads. The 8-peak beads are 3.2 µm particles excited by the blue laser. The beads emit light at eight different wavelengths. The validation of the benchtop flow cytometer is done by running the 8-peak beads and determining the coefficient of variation (CV) and mean fluorescence intensity (MFI) each time the instrument is used. These can also be plotted as CV and MFI in the Levy Jennings chart. The details of the quality control were described in our earlier publication [124, 125] and are also described in the manufacture's manual.

24.3.4.5 Running Kit Controls and Acquisition of Data for Kit Controls

The kit controls are run using the "Kit Control template" (◘ Figs. 24.3 and 24.4). The settings include "Run with limits" for a total of 10,000 events with fluidic speed set as "slow" and threshold set at 80,000 on FSC-H. Data is recorded on four plots: FSC-A/SSC-A, FSC-A/FL2-A, FL2-A/FL2-H, and FL1A/FL2-A. The FITC-positive values are recorded from the upper right quadrant as percent positive value for the negative and positive kit controls.

24.3.4.6 Running Patient Samples

Patient samples are run under the "Collect" tab using the standardized data acquisition template (◘ Fig. 24.5). The complete acquisition data should be saved in a designated folder for patient results. The steps involved in (1) running the sample and (2) data analysis which includes the "Analysis strategy in the collect tab" and "Data analysis in the analysis tab" are described below:

Running the Samples

1. Double-click on the folder for patient results "TUNEL patient template." Select well "F1" and import the standard sample file (.fcs) (◘ Fig. 24.5). This is the file for the "Standard sample" that is tested negative for SDF. The "Standard sample" is used as the internal reference to compare all samples being tested.
2. Select the first well "A1." In the space provided above the well, insert the file name as "TUNEL patient result, tech initials, date and well number." Click save button.
3. Begin with the first sample labeled as tube #1. Remove DI water tube from the SIP holder, vortex the sample, and place on the SIP holder.
4. Set the run parameters by selecting the following:
   1. "Run with limits": check "10,000 events"; "Fluidics" speed: select "Slow."

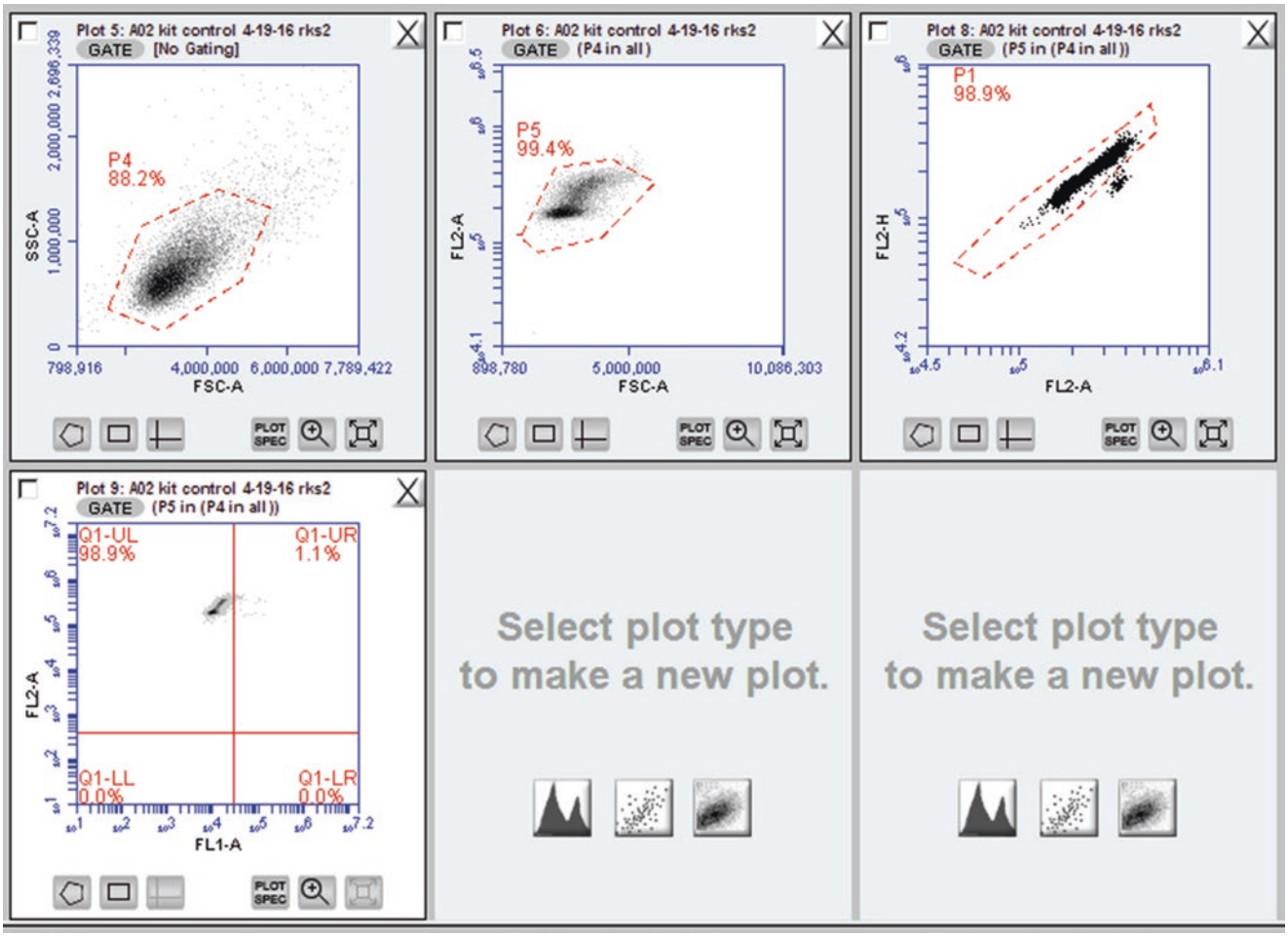

Fig. 24.3 Representative plot of "Negative kit control"

2. Select gate "P3 in P1" and set the threshold at 80,000 on "FSC-H."

5. Begin the acquisition of data by clicking on the "Run" button. The run is completed after 10,000 events.
6. Remove the tube from the SIP and clean the SIP using a lint free wipe. Vortex and place the second sample on the SIP.
7. Select the next well (A2, etc.) for the next samples. The above steps are repeated for each sample to allow processing of all samples.
8. Save the data acquisition workspace under a subfolder, e.g., "TUNEL Patient Results," "date," and "tech initials" C6. Save the workspace and close the file.
9. After all the samples are run, remove the tube from the SIP holder and replace with "bleach tube." Run the "Bleach cycle." A "Bleach cycle" is run to decontaminate the SIP holder and the fluidics using the following parameters:
   1. "Run with limits": 2 min.
   2. "Fluidics" speed: fast.
   3. Threshold: 80,000 on FSC-H.
10. After wiping the SIP at the end of the run, remove the tube and replace it with deionized water tube. Repeat (1–2) in step "D" above with deionized water, and shut down the instrument at the end of the run.

Data Analysis

Alignment strategy is performed under the "Collect tab." For this, the "Standard sample" file is used for alignment of all the samples. Data analysis is performed under the "Analyze tab," and each sample is aligned to "Standard sample" under the "Analyze tab."

Alignment Strategy and Data Analysis in the Collect Tab

The steps involved are described below:

1. Go to file, open workspace or template. Select the acquisition data saved in the TUNEL template (TUNEL patient template). Select an empty well where the standard sample data acquisition file has to be imported. Standard sample is a sample which has a known percentage of DNA fragmentation. Go to the "Standard template" and select it.
2. Click on "File import" and open the workspace.
3. Save the workspace as "TUNEL patient acquisition data analyzed, tech initials and date."
4. Select a single sample as "Standard sample."

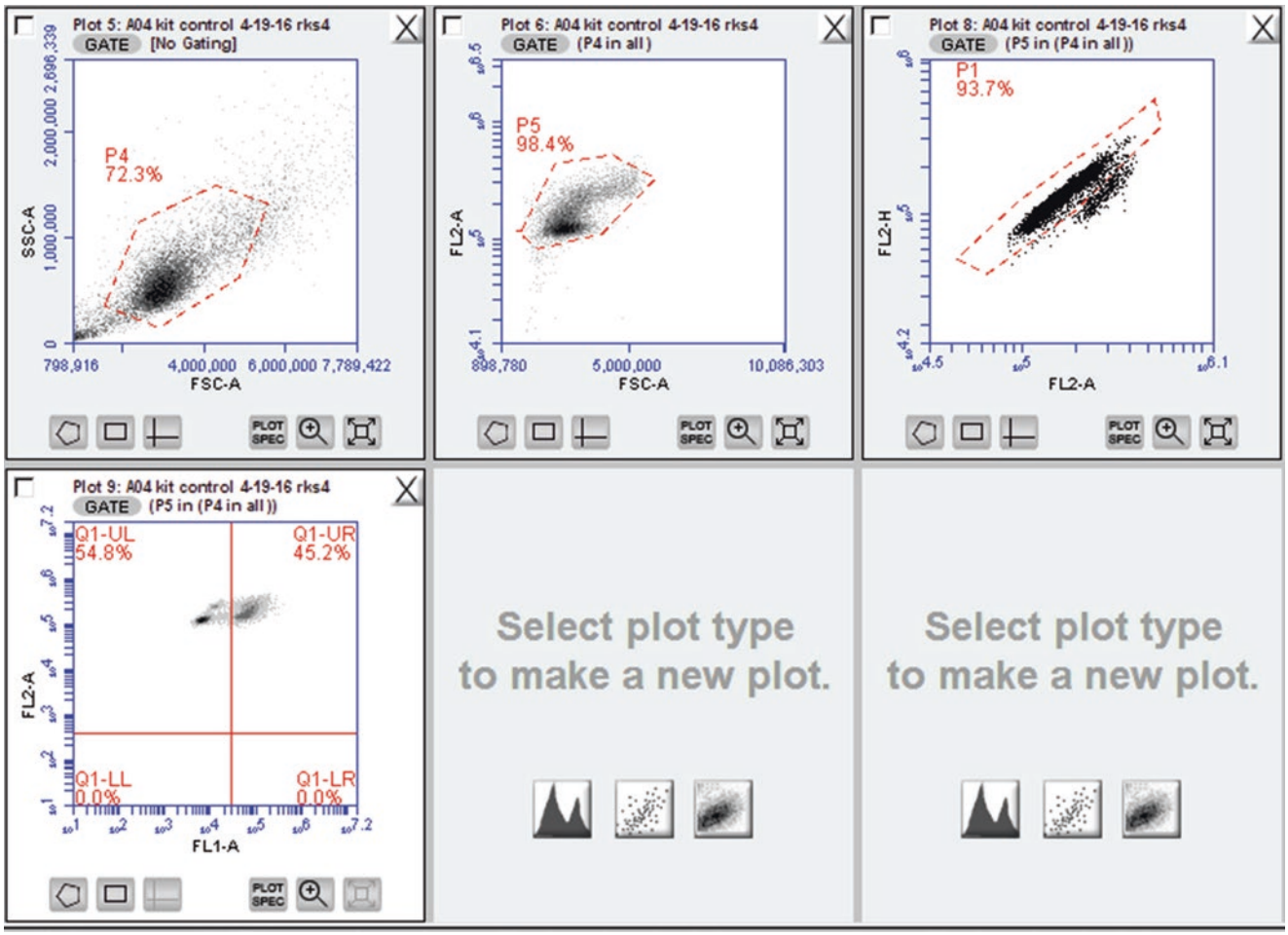

Fig. 24.4 Representative plot of "Positive kit control"

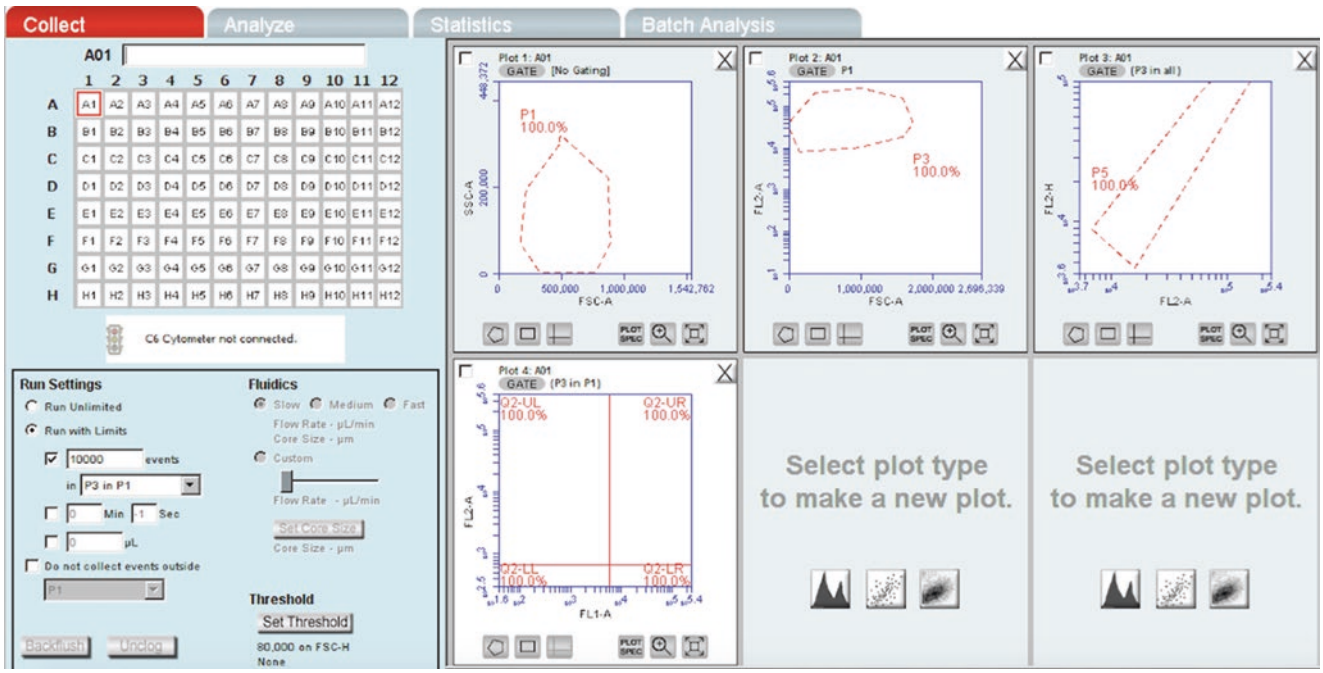

Fig. 24.5 Example of template setup for the analysis of the patient sample

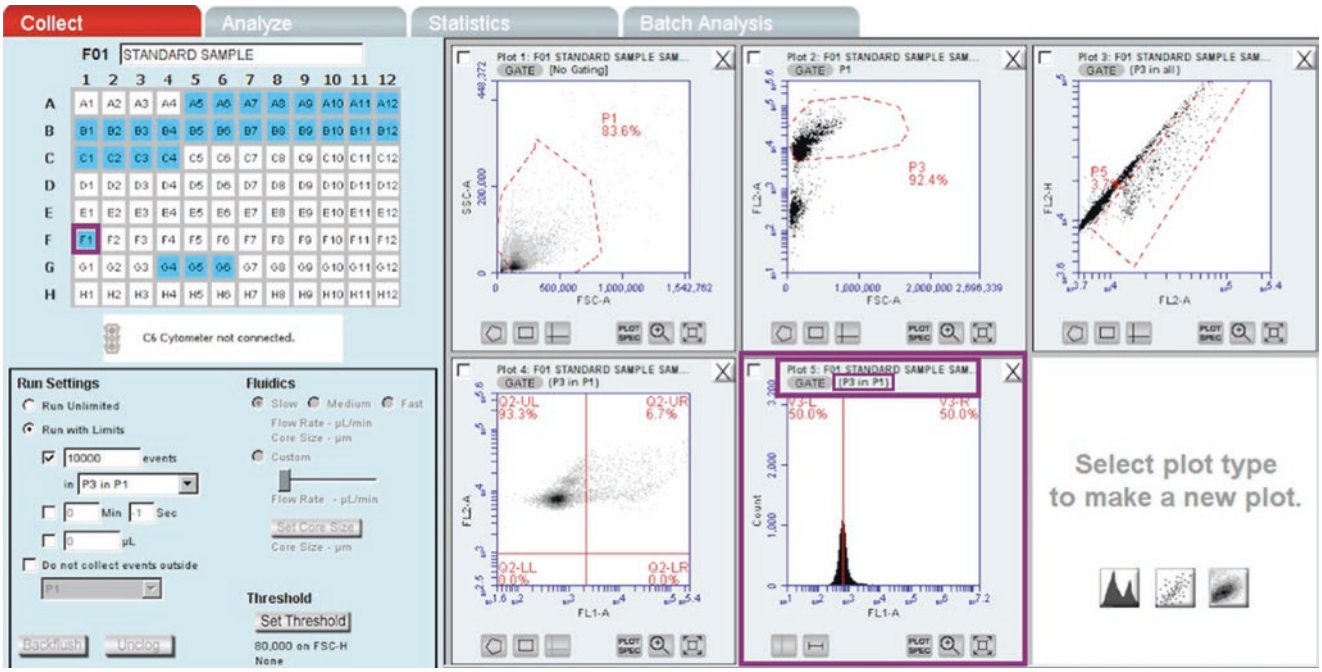

Fig. 24.6 Representation of a "Standard sample alignment"

5. Select the negative peak of the "Standard sample." This is used as a reference to be applied to all samples for alignment (Fig. 24.6).
6. Click on the histogram for the "Standard sample" and change the X-axis parameter from FSC-A to FL1-A.
7. Right-click below the X-axis (FL1-A) and select the "Virtual gain" module for alignment.
8. Change the gate to "P3 in P1" for plot 5. This gate is the same as plot 4, which is a quadrant gate.
9. Select the vertical line icon at the bottom left of the histogram plot, and align the selected blue line to the center of the histogram to obtain 50% cell population on either side (Fig. 24.7).
10. Select the sample to be aligned from the grid of wells.
11. Next align the blue line to the center of the peak of the selected sample. Click the tabs of "Preview" and "Apply."
12. An "Asterisk" will appear below the histogram. This confirms the alignment of the sample (Figs. 24.8 and 24.9).
13. Next hit close button. Go to file and hit "Save" after each sample is aligned.

Data Analysis in "Analyze" Tab

The data acquired in the "Collect" tab is utilized for analysis under the "Analyze" tab within the Accuri C6 software. After opening the "Analyze" tab, a set of three plots are created for each sample: FSC-A/SSC-A, FSC-A/FL2-A, and FL1-A/FL2-A. The same gating strategy that was used in the "Collect" tab is used here as follows:

1. The first plot "FSC-A/SSC-A" has no gating. The cell population is PX.
2. The second plot "FSC-A/FL2-A" will have the gate PX in all events. The cell population is PY.

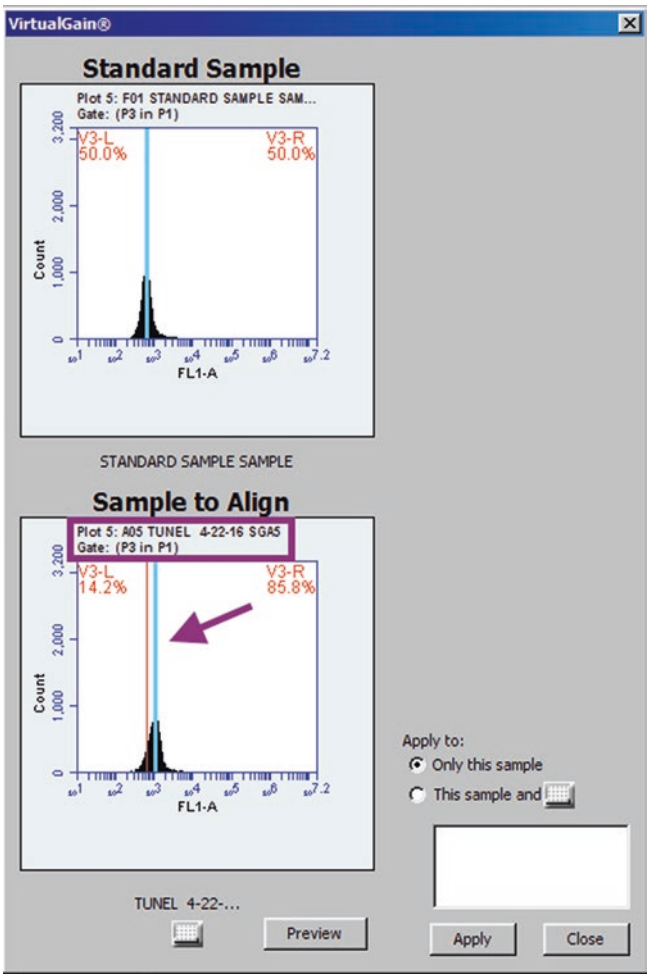

Fig. 24.7 Aligning a test sample to the standard sample

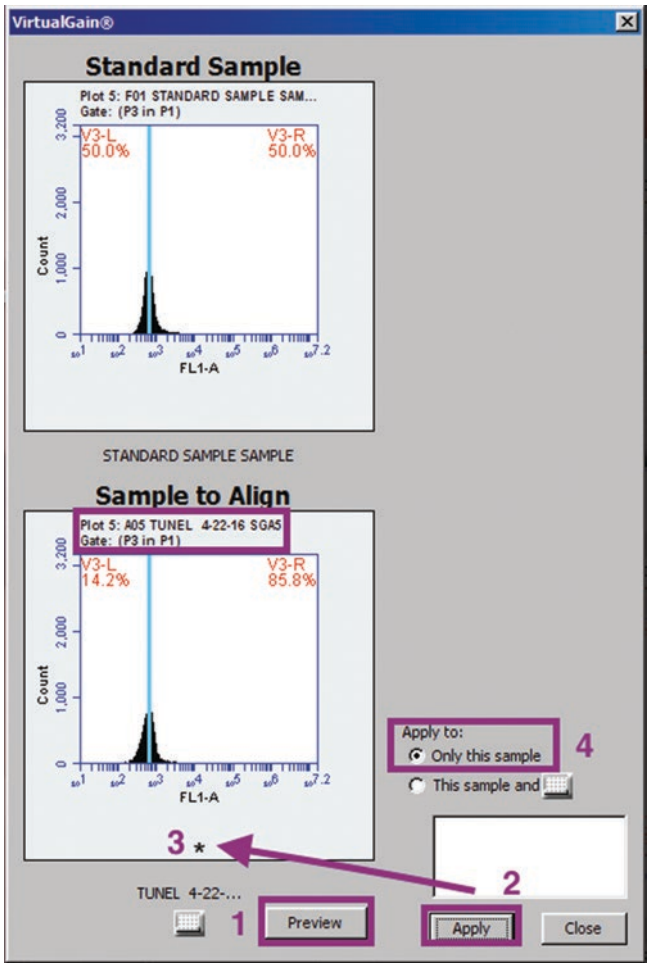

Fig. 24.8 Applying the alignment to the test sample. This is indicated by an asterisk at the bottom of the histogram confirming the alignment of the sample to the standard file

3. The third plot "FL1-A/FL2-A" will have gate of PY in PX in all events.
4. Record the percent damage reflected in the upper right quadrant from the "FL1-A/FL2-A" plot (Fig. 24.10).
5. Record the preliminary results in the "TUNEL Laboratory record form."

Calculation of Sperm DNA Fragmentation Result and Validation of the TUNEL Assay

After subtracting the average value of the "Negative samples" from the percent damage for each sample (Fig. 24.10), the assay is validated if the (1) "Positive control" spermatozoa samples have higher percentage of sperm that are positive for TUNEL assay than percentage of positive spermatozoa in the actual spermatozoa samples and (2) the "Positive kit control" has greater than 30% of spermatozoa positive for TUNEL assay. Currently, the reference value established in our center for SDF is 17%. Samples with SDF <17% are considered as "Normal," and those with >17% SDF are considered as "Abnormal." It is important that each laboratory establishes their own reference values and adopts strict quality control standards. The instrument quality control must be performed regularly using the 8-peak beads provided by the manufacturer. The "Positive" and Negative" controls provided in the kit and appropriate internal spermatozoa "Negative and Positive controls" must be included with each run. The details of the quality control steps are described in our recent publications [124, 125]. Adopting these strategies will help establish the reference values from different labs and increase the confidence of the results obtained. This will help in the clinical management of the patients.

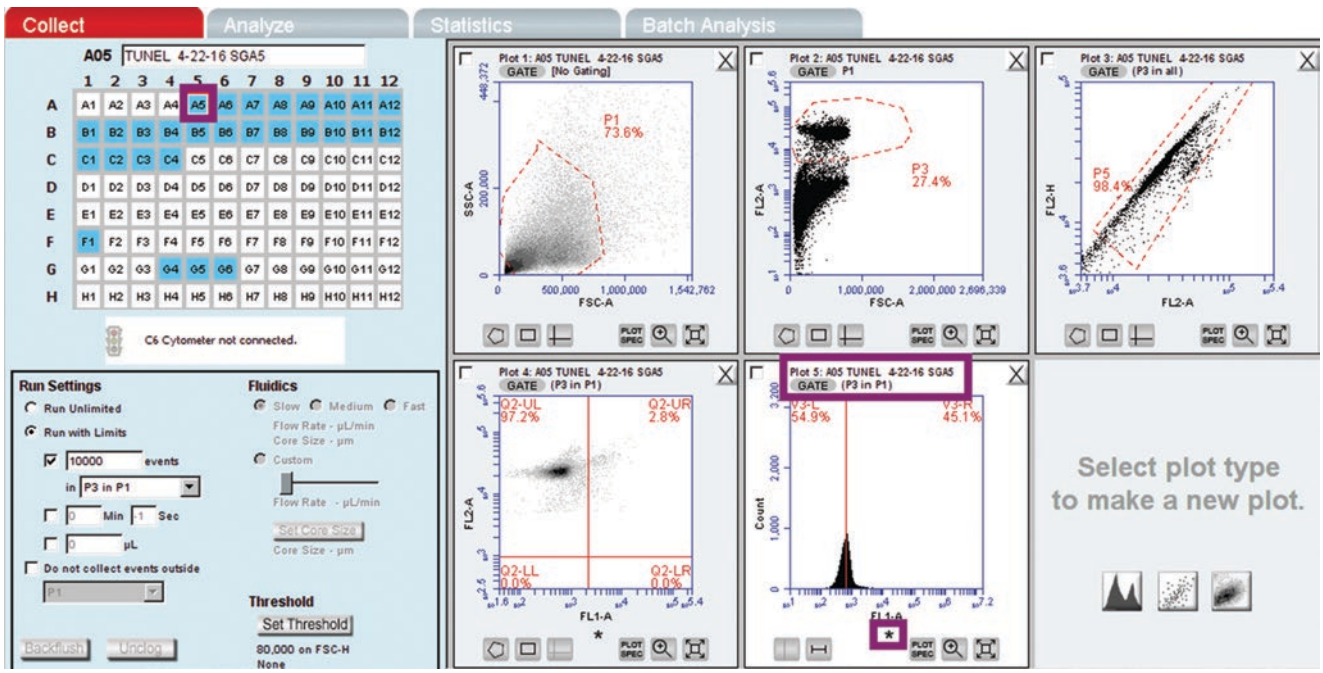

Fig. 24.9 Steps showing the alignment of the well with a star saved under the histogram plot

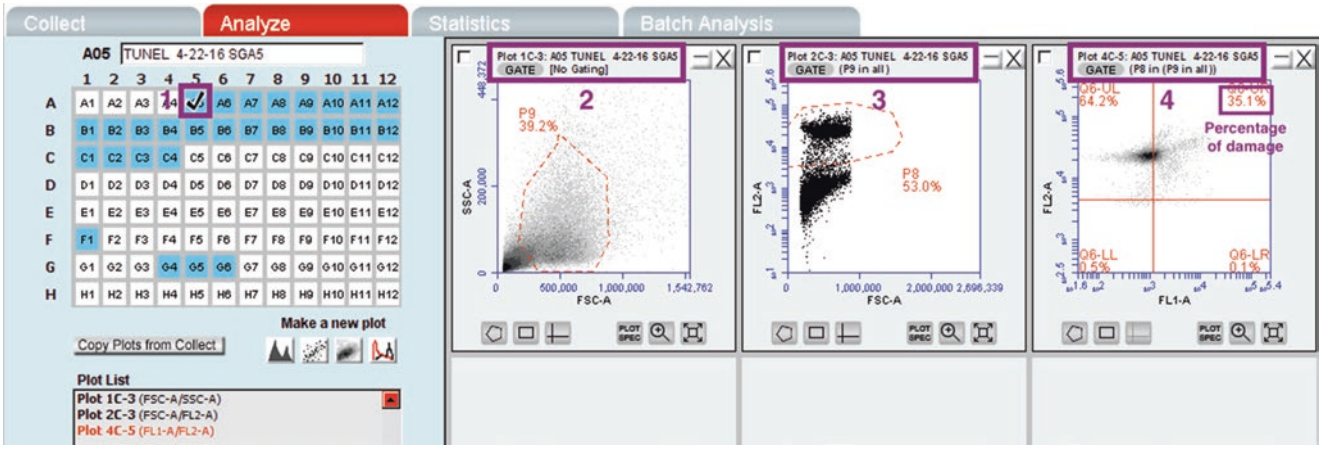

Fig. 24.10 Plot in the analyzed mode showing percentage of DNA damage

24.4 Conclusion

It is important that chromatin from the spermatozoa is correctly packaged and delivered safely to the oocyte. Studies have reported the association between the sperm DNA fragmentation and success rates of ART such as fertilization and pregnancy outcomes. Based on the extent of DNA fragmentation, the appropriate selection of ART is important to make it efficient and cost-effective. The assessment of DNA fragmentation can be performed using different techniques, still the clinical relevance of many tests remains unclear. Nevertheless, TUNEL assay is gaining popularity, and it is important that more laboratories standardize this test and establish the reference values. This is important to establish the guidelines for use of this test to be considered as a "gold standard" in a clinical setting.

Review Questions

1. In sperm DNA, during chromatin packaging the majority of the histones are replaced with:
   (a) Protamines
   (b) DNA topoisomerases
   (c) Disulfide bonds
   (d) None of the above

2. Which of these causes can be responsible for DNA fragmentation:
   (a) Aging
   (b) Lifestyle
   (c) Medical history
   (d) All of the above

3. In the TUNEL assay the samples can be evaluated by:
   (a) Flow cytometry
   (b) Standard fluorescence microscope
   (c) Polymerase chain reaction
   (d) (a) and (b)

4. In the TUNEL assay:
   (a) The intensity of labeling is not proportional to the number of DNA strand break sites.
   (b) The intensity of labeling is proportional to the number of DNA strand break sites.
   (c) There is no relationship between the intensity labeling and the number of DNA strand break sites.
   (d) None of the above.

5. What is the most important parameter in the sperm chromatin structure assay:
   (a) DNA fragmentation index
   (b) Number of DNA strand breaks
   (c) Sperm chromatin packing index
   (d) Protamine concentration

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Sperm Processing in Assisted Reproductive Technology

Rocio Rivera-Egea, Nicolás Garrido, and Alex C. Varghese

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Learning Objectives

- To provide a brief overview of the principles, purposes, and goals of processing of semen samples
- To discuss sperm-processing techniques and provide a step-by-step protocol, with troubleshooting notes
- To provide a literature review on the various sperm-processing and selection techniques that are best suited to different types of samples

25.1 Introduction

The use of sperm preparation techniques as a part of assisted reproduction procedures, including intrauterine insemination (IUI), was initially established to select motile and morphologically normal spermatozoa that are ready to be used in the clinical process and separate them from the debris of the ejaculate and from other cell types present in the ejaculate, such as leukocytes, bacteria, and dead spermatozoa, which produce reactive oxygen species (ROS) that may negatively influence fertilization, embryo development, and implantation [1–3].

Additionally—and mainly when the intended use of the ejaculate is IUI—seminal plasma (the fluid resulting from reproductive tract gland secretions) is removed during the procedure to avoid the unnecessary and harmful presence of (among other things) prostaglandins, which may cause involuntary uterine contractions or spasms. Unprepared semen has been demonstrated to induce pelvic inflammatory disease, infections such as endometritis, cervicitis, or vaginitis, and has been related to increased miscarriage, probably together with increased risks of premature delivery or fetal malformation [4, 5].

Historically the beneficial effect of preparing sperm in assisted reproductive technology (ART) has been well established, and significantly increases the probability of conception after IUI in couples with male subfertility [6], when sufficient spermatozoa are retrieved after the process of sperm selection.

The number of motile and normal spermatozoa after preparation will determine the treatments to be applied and the outcomes. There is a close relationship between the probability of conception after IUI and the absolute number of motile sperm inseminated, until a threshold is reached, beyond which pregnancy rates form a plateau [7–9]. These minimal numbers to maximize results may vary from one million to several million motile sperm. A high yield can lead to a preference for IUI or in vitro fertilization (IVF), whereas a lower yield may lead to a preference for intracytoplasmic sperm injection (ICSI).

These are the reasons why sperm preparation can also be considered, in a way, a diagnostic tool to assist practitioners and patients to select the most convenient ART, depending on the balance between the female factor findings in the initial work-up and the sperm quality (both before and after sperm preparation), mimicking the situation when the sample will be prepared for therapeutic purposes.

On the basis of our criteria (Fig. 25.1), which are based on our own experience and clinical results, we provide our patients with such recommendations to better decide the most convenient approach for them. Different sperm preparation methods are available; the following are the most popular and widely used ones:

1. In the "swim-up" (SU) method, spermatozoa are self-selected on the basis of their ability to swim. This technique involves first mixing culture medium with the liquefied semen, then removing the supernatant after centrifugation, and, finally, smoothly layering the culture medium over the cell pellet, which motile spermatozoa swim up into. After an incubation of 30–45 min, the upper part of the layered medium is then carefully separated for further use.
2. The second most popular method for selecting spermatozoa uses density gradients. In this case, the semen sample is pipetted on top of a column composed of a substance of known density, greater than that of the ejaculate, thus keeping it physically separate, and it is

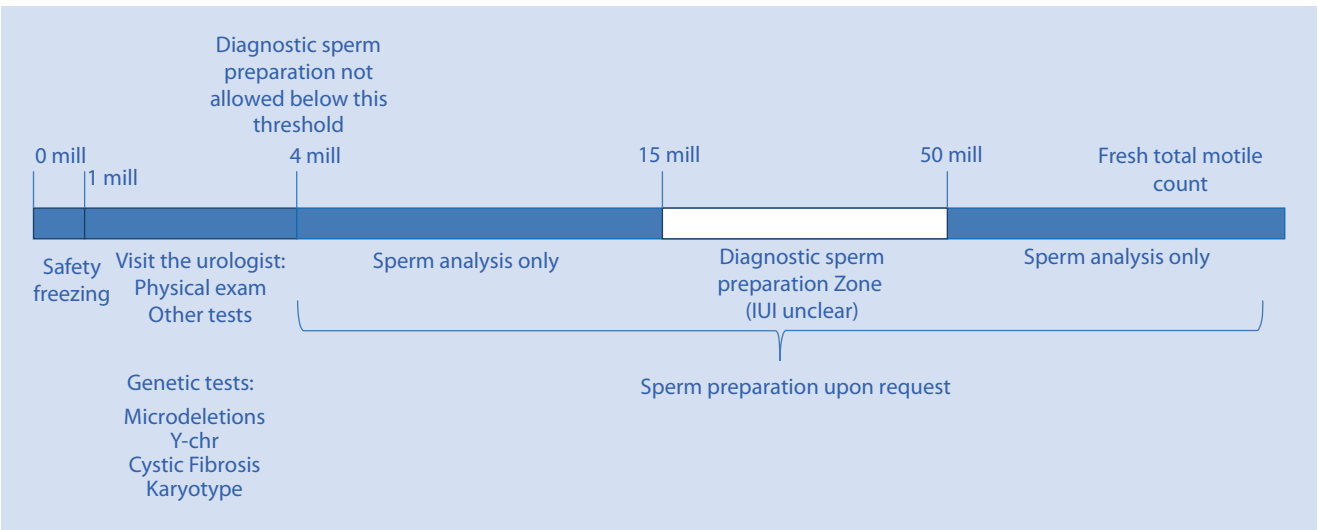

Fig. 25.1 Clinical recommendations and action patterns, depending on the spermiogram results. *Y-chr* Y-chromosome

then centrifuged. The centrifugal forces make motile sperm—and only motile sperm—cross through the density gradient, while keeping the remaining part of the ejaculate in the upper layers. Then, by just removing the upper layers or going through them with, for instance, the help of a Pasteur pipette, one can remove the motile sperm fraction to be washed from the gradients and finally resuspended in fresh culture medium [10].

Probably, these procedures are common to all ART units worldwide and are part of the essential procedures in each laboratory.

In natural conception, we understand very little about the characteristics of sperm populations that lead to reproductive success and what makes them do so. Despite decades of research, we still lack knowledge about the molecular features of optimal sperm and, logically, if we were able to identify or use these cells in ART, the success rate (the sperm to fertilized egg ratio) would be significantly increased.

To this end, instead of the self-selection methods described above, there are several direct selection methods being developed to retrieve sperm, with specific molecular features linked to optimal reproductive results. Unfortunately, many of these advanced sperm selection methods are still under investigation, while others are applicable only to a specific subset of patients. Thus, until further developments are available, there is a need to use classical sperm selection methods in the proper manner to optimize results.

The aim of this chapter is to describe the rationale, the procedures, and the most relevant aspects of routine sperm selection methods used in ART.

25.2 Overview and Essentials in a Sperm-Processing Unit

The minimal technical laboratory requirements and devices needed to run a sperm-processing unit—apart from a suitable building where the laboratory is located, with all of the architectural requirements depending on local regulations—include all of the resources that enable the possibility of obtaining sperm samples so they can be delivered safely to the laboratory, and also include places to conduct personal interviews, filing, semen analyses, and preparation.

Also, there is a need for equipment to perform blood, urine, and semen tests to confirm the absence of infectious conditions (as is often a prerequisite for any sperm preparation), although all of these aspects may be referred to external laboratories.

In this sense, the minimal list of requirements includes:

1. Laminar flow hoods to maintain and handle sperm samples under the most sterile conditions available and incubators with a continuous CO_2 supply to receive and keep the samples until liquefaction is completed properly, permitting (in some cases) sperm cell activity related to sperm preparation (as in the swim-up method). If media with HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid) or MOPS (3-Morpholinopropane-1-sulfonic acid) buffers are used, a warming incubator is required.
2. Bright-field microscopes to analyze the samples and centrifuges to concentrate sperm cells from raw ejaculate, which are needed in some sperm preparation procedures described later in this chapter.
3. Ideally, an electronic filing system, or at least a paper-based filing system, to keep all donor records available.
4. Reference laboratories to perform screening tests for both infectious and genetic disorders, if these tests are not run at the sperm preparation facility.
5. Generation and maintenance of standard operational procedures (SOPs) for each procedure and records of regular maintenance and preparation of all equipment.

Moreover, qualified personnel trained in assisted reproduction laboratory tests—including sperm preparation, quality assessment, and serological tests—are required, together with laboratory directors and technical and administrative personnel.

25.3 Sperm-Processing Media

Many publications have compared different sperm-processing techniques, as outlined below, and some publications have compared the various culture media used in each technique [11–20].

Ficoll® was initially used as a density gradient centrifugation (DGC) medium for preparing spermatozoa, but then Percoll® replaced it. During the 1990s, several different types of media were developed to replace Percoll®—given its risk of contamination with endotoxins, membrane alterations, and inflammatory responses—including IxaPrep® (MediCult, Copenhagen, Denmark), SilSelect® (FertiPro, Beernem, Belgium), PureSperm® (NidaCon Laboratories, Gothenburg, Sweden), and ISolate® (Irvine Scientific, Santa Ana, CA, USA). Nycodenz® medium (Nyegaard & Co., Oslo, Norway), used in DGC, was assessed and found to recover highly motile spermatozoa with a better yield and better survival than other media used for oligozoospermic and asthenozoospermic samples. The remaining media had very low toxicity, but the results of sperm processing using these media varied between laboratories. This fact can be attributed to different conditions of the methodology, making these data impossible to compare. However, the new products were suitable alternatives to Percoll®.

Allamaneni et al. [11] compared the efficacy of two of these available density gradient media—PureSperm® and ISolate®—in different densities of layers: 40–80% in the first instance and 50–90% in the second instance. They compared sperm characteristics and ROS levels after processing the sample with these two gradient media and demonstrated that sperm fractions prepared using PureSperm® had significantly higher total sperm counts and higher recovery rates than those prepared using ISolate®. Therefore, they concluded

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that higher rates of mature sperm recovery were obtained using density gradients—and, specifically, PureSperm®—which could be advantageous for ART success.

Nine years later, Malvezzi et al. [14] performed a controlled trial to study sperm quality after DGC with three commercial media: PureCeption® (Cooper Surgical, Trumbull, CT, USA), with 40% and 80% gradients; ISolate®, with 50% and 90% gradients; and SpermGrad-125® (Vitrolife, San Diego, CA, USA), with 45% and 90% gradients. For this purpose, they analyzed the percentages of sperm motility, total motile sperm, recovery, and deoxyribonucleic acid (DNA) damage, reproducing the same conditions. They found that although all three sperm-processing media produced good-quality samples, the ISolate® and SpermGrad-125® sperm fractions had higher percentages of sperm motility and total motile sperm than PureCeption®, with the percentage of DNA damage being comparable among the three media.

Similarly, several researcher groups assessed the efficacy of media—in this case, using sperm washing or the swim-up technique. In 1987, Vijayakumar et al. [20] assessed diverse wash culture media to maximize motile sperm recovery after the swim-up technique. They studied WT-6®, Ham's F-10®, and BWW®, and demonstrated that although the largest numbers of motile sperm were recovered with the first two media, the first one produced a higher yield.

Kim et al. [12] also compared the effects of different media but related them to clinical outcomes using the DGC/swim-up method or the swim-up method alone. In this case, they used Ham's F-10® medium or sperm wash medium. They concluded that using either culture medium, the results were comparable, including fertilization and pregnancy rates.

Later, several attempts to improve recovery of the largest number of motile sperm were made by several groups. The likelihood of achieving pregnancy was greater if the number of motile sperm was greater, leading Carlsson et al. [18] to include prostasomes in the swim-up medium when the semen was cryopreserved. They reported that prostasomes might be of benefit in improving outcomes in ART.

Likewise, aiming to avoid contamination in sperm processing for ART, another group [19] supplemented swim-up culture media with antibiotics (penicillin and streptomycin). They concluded that the combination of these antibiotics could remove nonspecific bacteria from semen samples without affecting sperm quality. A similar study was carried out 17 years earlier [15], and the researchers concluded that performing washing and swim-up preparation using culture medium rich in penicillin and streptomycin eliminated microorganisms with a 95% success rate.

Other studies have focused on searching nonanimal alternatives to avoid adding animal substances to culture media [21]. In one study, five different nonanimal macromolecules (Select Phytone™ UF, wheat peptone, dextran, hydroxyethyl starch, and methyl cellulose) were investigated for use as a supplement in culture medium for human sperm cells and compared with bovine serum albumin. However, it was concluded that although there were nonanimal macromolecules available for supplementing sperm culture media, some of them may be unsuitable for ART because of their physical properties.

The ideal option could be to supplement the sperm-processing medium with donor follicular fluid—a biological fluid rich in nutrients, growth factors, and hormones that might improve sperm quality. Following this theory, Bahmanpour [16] supplemented Ham's F-10® sperm medium with 10% follicular fluid, obtaining improvements in sperm quality in terms of survival and maintenance of chromatin integrity when the swim-up technique was used.

25.4 Human Semen Ejaculate

The words "semen" and "sperm" come from Latin (*semen* and *sperma*; the latter means "seed"). Sperm and semen constitute the set of spermatozoa and fluid substances that are produced in the male reproductive tract of all animals, including the human species. Semen is a viscous, slightly yellowish or whitish fluid, which is expelled through the urethra during ejaculation, usually accompanied by orgasm. Ejaculation is composed of two different actions: emission or deposition of seminal fluid from different glands into the posterior urethra, and ejaculation or propulsion of this fluid trough the urethra and out of the penis [22]. This fluid is composed of spermatozoa and seminal plasma. These two components of semen differ in their composition, function, and origin [23].

Seminal plasma is formed by the contributions of the testes, epididymis, seminal vesicles, prostate gland, bulbourethral glands (Cowper's glands), periurethral gland (Littré's gland), and vas deferens [24]. The function of this seminal plasma is to act as a nutrient medium, with suitable osmolality and volume to drive the spermatozoa to the cervical mucus for fertilization and to activate the spermatozoa to improve their motility.

The spermatozoa are formed by the testis from spermatogenetic cells of the seminiferous epithelium during spermatogenesis and are maturated in the epididymis. Their function is to reach the oocyte and fertilize it.

25.4.1 Semen Composition

During ejaculation, the sperm pass through the testes, epididymis, and ejaculatory ducts (vas deferens), and mix with components from the seminal vesicles, prostate gland, bulbourethral glands, and periurethral gland to form the semen. Generally, normal ejaculate has a volume of 1.5–5 mL and an alkaline pH of 7.0–8.5. Its composition depends on the proportions of sperm and plasma, and the size, storage capacity, and secretory output of several organs in the male reproductive tract [22, 23, 25].

25.4.1.1 Seminal Plasma

Seminal plasma is an extracellular fluid able to carry and feed spermatozoa. It represents 90% of the composition of semen. As mentioned earlier, its composition depends on the

secretion of different exocrine glands, each one contributing a different proportion and different components.

The prostate is positioned under the urinary bladder. It produces an opaque and whitish secretion that represents about 20% of the total volume of semen. Its pH is slightly acidic (6.2–6.8) [26] because its main components are citric acid, acid phosphatase, and for its reducing content in sugar. The prostate also secretes proteolytic enzymes, spermine, albumin, zinc, calcium, sodium, potassium, and lipids. The high content of zinc is important because it stabilizes chromatin in the sperm cells, and a deficiency of it can adversely affect spermatogenesis [27]; therefore, zinc plays an important role in reproduction. The main function of the prostatic secretion is liquefication of the sample by means of fibrinolysin, improving sperm motility and neutralizing the acidity of the other seminal fluids during ejaculation [27].

The seminal vesicles, located behind the prostate, secrete an opalescent, viscous, yellowish fluid, which contributes about 70% of the ejaculate fluid. Its pH is alkaline. It is composed mainly of fructose, prostaglandins, fibrinogen, lactoferrin, flavins, proteins, potassium, and bicarbonate, and it has a high content of reducing substances [28]. Its main functions are producing adenosine triphosphate (ATP), because fructose is the main energy source of sperm cells [29], and improving sperm motility and viability. Also, the prostaglandins may assist in fertilization, making cervical mucus more receptive to sperm and moving the ejaculate toward the ovaries.

The bulbourethral glands, or Cowper's glands, are located under the prostate. They secrete alkaline mucus composed of galactose and sialic acid, with a clear secretion into the lumen of the urethra to wash and lubricate it, facilitating the motility of the spermatozoa through the vagina and cervix to the ovaries [28]. These glands contribute about 10% of the ejaculate fluid. The periurethral gland (Littré's gland), located posterior to the urethral mucosa, also produces mucous fluid.

Therefore, human seminal plasma contains a complex range of organic and inorganic constituents, providing a nutritive and protective medium for the spermatozoa during their passage through the female reproductive tract toward the oocyte. Moreover, as mentioned earlier, the alkaline components of the semen compensate for the aggressive environment of the vagina toward the spermatozoa, given that this acidic environment could damage sperm DNA and cause sperm denaturation.

25.4.1.2 Spermatozoa

Less than 10% of the total volume of the ejaculate consists of spermatozoa—from 1% to 5%. The number of spermatozoa can generally vary from 50 million to 150 million per milliliter [30, 31]. The spermatozoa originate in seminiferous tubules inside the testes, then they are maturated in the epididymis to be conducted by the vas deferens through the seminal vesicles and the urethra to the penile meatus [22].

Spermatogenesis, or the sperm cell differentiation process, starts with spermatogonia and finishes with mature spermatozoa [28].

In the first step, spermatogonia migrate among Sertoli cells. These cells secrete a fluid into seminiferous tubes with substances necessary for their nutrition and development. Among these substances are transport proteins such as sex hormone–binding globulin (SHBG) and others, proteases and antiproteases, growth factors, other proteins such as inhibin and clusterin [32, 33], and another type of cells—Leydig cells—which produce testosterone, an essential hormone for sperm generation. During their course through the Sertoli cells, spermatogonia are progressively modified into spermatocytes, which undergo meiosis into secondary spermatocytes, then they are differentiated into haploid spermatids to become spermatozoa.

After spermatozoa are formed, they need to mature to develop the capability for motility and fertilization. For this purpose, they pass through the epididymis in their course from the testis to the vas deferens, where they mature. The epididymis secretes carnitine and alpha-glucosidase for sperm maturation, and motile inhibitory proteins to avoid premature capacitation until after ejaculation [22, 28]. Finally, they remain stored in the epididymis and vas deferens until ejaculation. After ejaculation the sperm acquire mobility and the capacity for fertilization.

25.5 Patient Communication and Preparation

Regarding communication with patients, there are two relevant situations during sperm preparation. The first one concerns the instructions to obtain and deliver the sample to the laboratory. These are the same as the actions requested for providing samples for basic semen analysis, and they can be found in the World Health Organization manual for semen analysis [34].

Another level of communication with patients concerns reproductive counseling after sperm analysis and preparation have been conducted. There is little or no published information about how to deal with the results of the prepared sperm analysis, regarding the sperm count and morphology. That is why, on the basis of our own experience, we have developed our own decision-making chart.

Briefly speaking, when preparing sperm from a diagnostic perspective, it is necessary to determine how the sample will perform functionally on the day when it will be needed for an ART procedure.

Several questions may be raised in this regard. First, in the event that the sample is aimed at IUI, the question is whether there are sufficient spermatozoa with optimal motility to maximize the chances of reproductive success. According to our own data, performing IUI with at least five million total motile cells after preparation will maximize the chance of pregnancy per insemination.

Anything less will, to some extent, hinder that possibility, and this situation should be discussed with patients, to take an agreed decision on the suitability of going ahead with this process. Our own experience and others' show that the

fewer motile sperm cells available, the lower the chance of pregnancy.

The second question that may be raised regarding the decision between classical IVF or ICSI depends on the total motile count available after sperm preparation.

Although the criteria may differ between laboratories, ejaculates in which the recovery rates are below 3–4 million are typically directed to ICSI. Patient also need to be counseled for prior cryo-storage of 1–2 ejaculated in case the husband couldn't collect the sample on the day of egg recovery. Second, patient also need to be informed of the benefit of frequent ejaculations prior to the day of IVF or repeat sample on the day of procedure. Such samples have shown to have improved DNA integrity in cases of high Sperm DFI.

25.6 Laboratory Documentation

Laboratory documentation includes SOPs and consents.

25.6.1 Standard Operating Procedures

Uniformity in all procedures that are carried out in a laboratory is an essential aspect for maintenance of a good level of execution of different methods in clinic embryology. Such uniformity allows us to confirm that all personnel perform techniques in a specific and coherent way, and it facilitates the identification of possible mistakes.

For this reason, the elaboration of SOPs is essential. They are detailed written instructions designed to achieve uniformity in the performance of specific functions. They are a set of step-by-step instructions compiled by an organization to help workers carry out routine operations to achieve efficiency, quality, and uniformity of performance, reducing miscommunication and failure to perform techniques. Moreover, SOPs facilitate the training of new workers and help personnel to remember procedures that are performed infrequently.

These SOPs should be passed on without modification, and if a modification is needed, it should be assessed and approved by the director of the laboratory, generating a new version of the SOP.

This documentation should be kept in the laboratory, and in a place accessible to all workers. The text should be reviewed by the director of the laboratory to change or update aspects, bearing in mind that the existence of these documents is referred to in good laboratory practice and in the International Organization for Standardization (ISO) standard ISO 15189:2003 [35–37].

Given that the elaboration of SOPs is very laborious, there exist different guidelines for how to write this type of documentation [38]. These are certain points that these documents must cover:

- The reason why the procedure is performed
- The principle on which the procedure is based
- If applicable, any specifications about precision, accuracy, etc.
- The type of sample used in the procedure
- Type of candidate suitable for the procedure
- The types of devices needed
- The type of calibration needed
- A description of procedural steps
- Quality controls
- Warnings about possible disturbances
- Expected results
- Information regarding interpretation of results
- Biosecurity warnings
- Any sources of variability

Besides a description of laboratory procedures, the SOP should have indicators that enable us to know if procedures have been performed correctly or not. Therefore, the SOP should contain solutions for each possible accident or mistake. Also, the SOP should be as detailed as possible, so that it would enable a worker from elsewhere to perform the technique without problems or doubts.

25.6.2 Reproductive Health Care Consent

Health care providers must obtain prior consent to treat patients [39], to ensure protection for both the patients and the providers. For this purpose, and to avoid problems, patients need to understand the informed consent that is required. If a patient cannot understand the consent, a legally recognized substitute or judge can provide it, considering that negligence could arise if the consent is not adequately informed. There may be legal limits to the reproductive procedures to which patients may consent, under laws that can be respectfully tested but must be obeyed.

Reproductive medicine professionals explain and confirm correct fulfillment and understanding of all relevant procedural consent requirements. Consents exist for all such treatments—from simple sperm-freezing preservation to the oocyte donation cycle or any analysis.

25.7 Decision Making Prior to IVF/ICSI: What Procedure to Use for Individual Samples

In the majority of patients undergoing IVF/ICSI, the spermatozoa are isolated from fresh ejaculate provided on the day of egg retrieval. For a sample to qualify for conventional insemination, it should yield a good number of sperm with excellent, lasting, and progressive motility postprocessing. As per the classical textbooks, typically 50,000 to 100,000 normal motile spermatozoa per milliliter are used for insemination. There can be variation in the insemination concentration depending on the culture volume (droplets or 4-well dishes/center well), sperm quality (especially in cases of teratozoospermia), etc. Occasionally, higher insemination volumes may be required with short-incubation IVF. Contrary to this, if the insemination is being done using ICSI, the final yield might contain more debris and not be as good and as neat as a conventional insemination case. In ICSI insemination, embryologists prefer

Table 25.1 Recommended sperm-processing/selection methods, depending on the type of sample or case

Type of sample/case	Recommended method
Normozoospermia	DGC/MFSS
Teratozoospermia	DGC/MACS
Asthenozoospermia	DGC/MACS
Oligoasthenoteratozoospermia	Wash and swim-up/DGC
Leukocytospermia	DGC/MACS
Hyperviscosity	Pellet swim-up
Hypospermia	Wash and pellet
Heavy debris	DGC
Retrograde ejaculate	Wash and pellet
PESA sample	DGC/wash
TESE sample	Wash and pellet/DGC
M TESE sample	Wash and pellet/DGC
Frozen–thawed surgical sample	Wash and pellet
Frozen–thawed ejaculate	DGC
Sperm with a high DFI	DGC/MACS
Previous ICSI failure	IMSI/MACS/PICSI
Unexplained infertility	MACS/IMSI/PICSI
Previous RIF	MACS/PICSI
Previous failure of conventional insemination	DGC/MACS
Patient preference	As per discussion/ counseling

DFI DNA fragmentation index, *DGC* density gradient centrifugation, *DNA* deoxyribonucleic acid, *ICSI* intracytoplasmic sperm injection, *IMSI* intracytoplasmic morphologically selected sperm injection, *MACS* magnetic activated cell sorting, *MFSS* microfluidic sperm sorting, *M TESE* microdissection testicular sperm extraction, *PESA* percutaneous epididymal sperm aspiration, *PICSI* physiological intracytoplasmic sperm injection, *RIF* repeated implantation failure, *TESE* testicular sperm extraction

to get a smaller concentration with no debris, which makes the injection process smoother and is less time consuming. Some laboratories use a short centrifugation of only 1–2 min after the density gradient step. This helps to minimize massive accumulation of sperm cells in the bottom of the tube, especially in cases of normozoospermia (Table 25.1).

25.7.1 Utility of Electronic Medical Records

Electronic medical records (EMRs), which are very common in IVF clinics, are a handy tool for embryologists to consider the previous seminal and IVF/ICSI history of the patient. The embryology laboratory sheet used on the day of egg retrieval may include a section including options such as "IVF–ICSI–IVF/ICSI" to choose from. In some laboratories, the embryologists/laboratory managers discuss the preferred type of insemination on the basis of the sample quality and the patient's preferences in their discussion with the couple after egg retrieval. Given recent media reports on neonatal outcomes in children conceived through ICSI, there may be concerns for patients about the types of insemination the laboratory chooses. So, it is appropriate to have a discussion with the couple regarding their preferences for the insemination after egg retrieval. The laboratory can explain the pros and cons of each type of insemination procedure. It is also a good idea to record a note in the male/female notes section of the EMR about the outcome of the discussion regarding the insemination type being used. During counseling sessions, embryologists obtain relevant information such as whether the woman has previously had a natural pregnancy or a pregnancy through IUI cycles, to make sure that conventional insemination will not yield failed fertilization.

The use of density gradient–based semen processing for separation of a fraction containing the most normal and mostly motile sperm has been the method of choice in most ART laboratories. This practice has become part of the SOPs of so many ART laboratories that other types of sperm preparation methods are no longer practiced in many laboratories. However, some other methods still have a place in the processing of particular types of semen samples.

The physical characteristics of certain types of semen samples may interfere with the processing technique that is used. Hyperviscous samples pose a problem if the density gradient method is used. Such samples and those with liquefaction problems may require alternative steps. Mechanical liquefaction using an 18-gauge needle used to be a common practice, but this can hamper the functional capacity of spermatozoa. One recent study compared DNA fragmentation in human sperm samples with reduced, physiological, and increased viscosity to evaluate whether the process used to reduce the viscosity (expulsion of semen through a needle and syringe) significantly altered sperm DNA fragmentation. There was no difference in the DNA fragmentation rates between samples with reduced, increased, and physiological viscosity; however, the physical process of expulsion of semen through a syringe and needle did increase sperm DNA fragmentation [40].

Samples that are not liquefied properly may be combined with the sperm preparation medium and mixed well, or can be added to α-chymotrypsin (which is available to andrology laboratories in ready-to-mix vials) to reduce the viscosity. This can be followed by the density gradient step. Sometimes a wash and swim-up method is the method of choice for hyperviscous samples, as density gradients can trap sperm between the gradients. Honea et al. found that limited proteolysis using α-chymotrypsin in the treatment of hyperviscosity was effective in an in vitro setting such as IVF or IUI [41, 42].

Samples with excessive numbers of nonreproductive cells (e.g., epithelial cells, round cells, etc.) can be diluted with buffered sperm-processing medium and fractionated into

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multiple tubes for density gradient processing to get a better yield. In contrast to IUI sperm processing, the idea of this type of processing is to get a fraction of functionally good spermatozoa. Similarly, samples with many jelly particles can interfere with a good yield in density gradient processing. These samples should be diluted with buffered medium and kept for a few minutes so that the jelly particles settle down. The supernatant is then removed for DGC.

Unlike sperm function testing, which has been used only as a research tool for many decades because of its questionable relevance to ART, the sperm DNA fragmentation index (DFI) has made a successful entry into this field and has become an integral part of the infertility (male factor) workup. This is exemplified by the fact that the sperm DFI has been extensively correlated with embryo quality, pregnancy outcome, and miscarriage [43, 44]. Recently, many sperm selection techniques have been added to the armamentarium of ICSI. These include intracytoplasmic morphologically selected sperm injection (IMSI), physiological intracytoplasmic sperm injection (PICSI), magnetic activated cell sorting (MACS), microfluidic sperm sorting (MFSS), and microelectrophoretic sperm separation. Many prospective and retrospective studies have been done on the efficacy of these techniques and their outcomes. Adoption of some of these techniques seems a better option in ICSI because of the following realities: (1) ICSI for human treatment was introduced in the early 1990s as a technology before it became a science; (2) spermatozoa are selected randomly by a technician (with no component of natural selection); (3) the ART field is still searching for better treatment for male factor infertility; (4) the incidence rates of sperm DFI/sperm epigenome issues and "unexplained infertility" are increasing; and (5) there is still the problem of "what next" to offer in ART clinics when patients experience repeated failures.

MFSS devices have been tried at many clinics in recent times as an alternative to conventional processing techniques. A recent study [45] found significantly lower sperm DNA fragmentation rates with MFSS compared with centrifugation and the swim-up procedure. The microfluidic system can effectively separate raw semen samples into immobile and mobile groups without the need for centrifugation steps, at the same time avoiding damage to sperm, as single or multiple centrifugation steps damage spermatozoa via generation of ROS [46].

Similarly, MACS is a sperm preparation technique that isolates nonapoptotic spermatozoa on the basis of expression of phosphatidylserine in the membrane of apoptotic sperm. A systematic review and meta-analysis of prospective randomized trials by Gil et al. [33] showed that sperm selection using MACS resulted in statistically significant differences in pregnancy rates when compared with the DGC and swim-up techniques. Another study concluded that the MACs technique is a simple, noninvasive technique that can efficiently reduce DNA fragmentation in infertile patients with a varicocele prior to ART.

Human sperm that express hyaluronic acid receptors and bind to hyaluronic acid have a normal shape, minimal DNA fragmentation, and a low frequency of chromosomal aneuploidies. Use of hyaluronic acid–binding assays in ICSI cycles to improve clinical outcomes has been studied by many groups. In a recent systematic review and meta-analysis [47], use of a hyaluronic acid–binding sperm selection technique yielded no improvement in fertilization and pregnancy rates. Meta-analysis of all available studies showed improvements in embryo quality and the implantation rate, but when the analysis was restricted to prospective studies, it showed an improvement only in embryo quality. According to the authors, this analysis did not support routine use of hyaluronic acid–binding assays in all ICSI cycles. They concluded that further study is needed to identify patients who might benefit from this technique.

Majumdar et al. [48] examined whether injection of hyaluronic acid–bound sperm helps to improve outcomes in patients undergoing ICSI with unexplained infertility despite normal semen parameters. There were no differences in the fertilization rates, numbers of top-quality embryos, and clinical pregnancy rates between patients treated with ICSI and those treated with PICSI. However, a higher pregnancy loss rate (PLR) was observed in the ICSI group than in the PICSI group, although the difference was not statistically significant. In another study by Mokánszki et al. [49] the PLR was significantly lower with PICSI than with ICSI.

The diversity and the constant development of novel promising techniques to improve ICSI, in terms of spermatozoon quality, are certainly worth pursuing. Most of the techniques that have been discussed are still a long way from being established as routine practice in standard IVF laboratories. In most cases an experienced embryologist can achieve the same results. Although some of the techniques show great benefits, there is a need for large-scale, multicenter, randomized, controlled studies to quantify their importance before horizontal application can be suggested. Taking into consideration the a priori invasive nature of ICSI, when clinical application becomes a possibility, we need to proceed with caution and ensure that in the pursuit of innovation we are not sacrificing safety and the balance of the physiological and biological pathways of the spermatozoon's dynamic [50].

Finally, in cases of akinetozoospermia, the use of functional tests, such as a pentoxifylline test, a hypo-osmotic swelling (HOS) test, or, to a lesser extent, the laser touch technique, makes selection of viable spermatozoa easier.

Assessment of sperm morphology has been reconsidered since 2001, with the development of motile sperm organelle morphology examination (MSOME). This observation technique, which combines high-magnification microscopy and Nomarski interference contrast, makes it possible to select spermatozoa with as few vacuoles as possible before microinjection into the oocyte (via IMSI). More than 10 years after the development of the IMSI technique, the indications for IMSI and its ability to increase pregnancy and/or birth rates (in comparison with conventional ICSI) are still subject to debate. In an attempt to clarify this debate, Boitrelle et al. [51] performed a systematic literature review according to

the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. Given the scarcity of head-to-head IMSI-versus-ICSI studies, the only confirmed indication for IMSI was recurrent implantation failure following ICSI. According to the authors, all other potential indications for IMSI require further investigation. In one study comparing the two sperm selection techniques, there were no differences in high-magnification morphology and hyaluronic acid binding in the selection of euploid spermatozoa with intact DNA [52].

Pharmacological sperm stimulation has been studied in surgically retrieved sperm samples and ICSI. Ebner et al. [53] evaluated whether the use of theophylline improves sperm motility and treatment outcomes in frozen–thawed testicular sperm extraction (TESE). All but one patient (98.5%) showed a significant improvement in testicular sperm motility when theophylline was employed as a motility enhancer. In addition, sperm selection by an embryologist during ICSI took significantly less time in cases where theophylline was used than in those where it was not used. The corresponding rates of fertilization (79.9% versus 63.3%), blastulation (63.9% versus 46.8%), and clinical pregnancy (53.9% versus 23.8%) were significantly increased with the use of theophylline.

The presence of a very high concentration of erythrocytes together with few sperm sometimes results in a much longer searching period and blockage of injection needles. Use of large-diameter ICSI needles is an option in these situations to pick up the sperm from red blood cell (RBC)–laden drops and place them in fresh polyvinylpyrrolidone (PVP) drops after a washing step in HEPES/MOPS drops. Once enough sperm are collected, the operator can change the needle and use regular ICSI pipettes for sperm immobilization and injection.

One option for testicular biopsy specimens is suspension in HEPES/MOPS–buffered medium [54]. This can then be centrifuged for 5 min at 300 × *g*. Treatment of the testicular sperm pellet with erythrocyte-lysing buffer is performed by resuspending the testicular sperm pellet in 2–4 mL of erythrocyte-lysing buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 2 mM ethylenediaminetetraacetic acid (EDTA); pH 7.2) for 10 min at room temperature. Following this step, 5–10 mL of HEPES/MOPS–buffered medium supplemented with human serum albumin (HSA) is added to the suspension in the same tube and then centrifuged for 10 min at 500 × *g*. The resulting pellet is then resuspended with 1.5 mL of sperm wash medium. This resuspended pellet can be centrifuged again at 500 × *g* for 5 min. After centrifugation, the pellet is resuspended in 50 μL of sperm wash medium and used for ICSI.

The sperm membrane undergoes extensive surface remodeling as it matures in the epididymis. During this process, the sperm is encapsulated in an extensive glycocalyx layer, which provides the membrane with its characteristic negative electrostatic charge. In a recent study, Simon et al. [55] developed a method of microelectrophoresis to isolate sperm with a high negative membrane charge. The results suggested that selection of negatively charged spermatozoa (NCS) at a lower current may be an important biomarker to select healthy sperm for ART.

When sperm were picked according to their charge and analyzed directly, sperm DNA damage was lower in the NCS population (3.9% ± 1.5%) than in the control population (17.3% ± 3.2%) and the positively charged spermatozoa (PCS) population (27.8% ± 6.0%). The percentage of NCS was positively associated with the fertilization rate (r^2 = 0.469) and blastocyst development (r^2 = 0.308), and inversely associated with embryo arrest (r^2 = −0.253). The implantation rate and clinical pregnancy rate were higher in patients with a higher proportion of NCS.

25.8 Sperm-Processing Techniques

Sperm processing or preparation increases the potential for sperm fertility through different techniques performed in the laboratory to separate immotile spermatozoa, debris, and seminal plasma, and to select motile spermatozoa and male germ cells [14].

Sperm processing results in modifications of flagellar movement and head beating of the spermatozoa, facilitating their oocyte penetration. For this purpose, spermatozoa have to undergo the acrosome reaction, in which they release lysis enzymes enabling them to penetrate the zona pellucida. In this process, the sperm acrosomal membrane starts to merge with the external acrosomal membrane, leading to vesiculation and generation of pores through which enzymes are released.

Therefore, sperm processing is related to the facility of achieving sperm fertilization ability [56].

Before any ART, all samples should be processed by means of sperm processing or preparation techniques to select spermatozoa with the best motility and eliminate seminal plasma (with prostaglandins and motility inhibitors) and immotile spermatozoa, immature cells, and debris.

To determine the most optimal sperm preparation method for ART, different groups have studied diverse techniques for this purpose: the swim-up technique or migration method, swim-down, direct swim-up, DGC, sucrose, glass wool filtration, washing, and sperm concentration [57–59]. Today, an array of different methods is available, and the selection depends on the quality of the ejaculate. The ideal sperm-processing technique is one that is quick, easy, and cost effective; recovers a large number of motile sperm; does not cause sperm damage; removes immotile and dead sperm and other cells; eliminates toxic substances; and allows processing of larger volumes of ejaculate [14]. For these reasons, the swim-up and DGC techniques are the methods most often used in infertility laboratories because of their efficacy, simplicity, and good results in terms of pregnancies.

These techniques can be used together or separately. Several articles have concluded that use of them together is better [57, 60, 61]; however, other papers have reported the opposite [58, 62]. Despite differences in the percentage recovery of motile sperm with the different techniques, the

clinical outcomes, including fertilization and pregnancy rates, seem to be comparable [12, 63–65].

Going further, if we take into account which technique allows us to recover spermatozoa with the lowest rates of DNA fragmentation or apoptosis, although the differences between the swim-up, DGC, and DGC/swim-up methods were not significant [58, 61, 62, 66], the swim-up method was the best option for treatment of semen samples during IVF/ICSI, given its low cost and reduced time requirement [58]. Focusing on the sperm telomere length (STL), Zhao et al. [61] compared the effects of the DGC and swim-up procedures on STL, and the results showed that both methods could recover a sperm population with longer STL and better DNA integrity for ART. However, another group concluded that the choice of method depended on whether IVF/ICSI or IUI was to be performed, because they observed no functional differences between the spermatozoa selected by either method [66, 67].

Therefore, each laboratory employs the most suitable technique according to its needs.

25.8.1 Density Gradient Centrifugation

This technique is based on the ability of motile sperm to cross layers with different densities to reach the bottom of the tube, and seminal plasma, round cells, immature cells, debris, and nonprogressive spermatozoa are then discarded [68]. With this technique, the spermatozoa are separated on the basis of their density, as normal mature spermatozoa have slightly higher density than abnormal immature ones. Therefore, after centrifugation, each spermatozoon is placed at the gradient level that matches its density, and highly motile spermatozoa are at the bottom of the tube, forming a pellet [11]. This technique allows us to recover, in the best way, motile spermatozoa from oligozoospermic samples, asthenozoospermic samples, and samples with large numbers of cells and large amounts of debris [65, 69].

In fact, 20 years ago it was believed that DGC was the best technique for selecting sperm with normal morphology [70] and produced final spermatozoa pools with higher percentages of motility—and higher motility quality within all concentration ranges—than the swim-up technique [71].

The different steps in this procedure are:

1. Sliding a certain volume of culture medium impregnating the walls to the bottom of the tube, always starting with the highest-density layer.
2. Adding the same volume of gradient with a lower density than the previous one, placing it in the same way as in step one over the first bottom layer to avoid breaking the interface and avoid mixing the two layers. This volume and the sperm sample volume will depend on the sperm concentration and motility in the sample, from 0.5 mL to 1 mL.
3. Placing the same certain volume of semen into the tube, making it slide over the tube walls.
4. Centrifuging the tubes at 1500 revolutions per minute (rpm) for 10–20 min.
5. Recovering the pellets from all of the tubes and placing them in a new tube or removing all of the supernatants and placing all of the pellets together.
6. Washing this big pellet with a wash culture medium at 2200 rpm for 5–10 min, removing the supernatant, and resuspending the pellet with 0.5 mL of culture medium for ART.
7. Assessing the quality of the sample before it is used.

The density gradient may differ depending on the laboratory and the culture medium applied [70]. For example, if we use SpermGradient® (Cook Medical, Castletroy, Ireland), the layers will be 80% and 40%; if we use PureSperm®, the layers will be 80% and 40%; if we use ISolate®, the layers will be 50% and 90%; and if we use SpermGrad®, the layers will be 90% and 45%. Other culture media are PureSperm® 100% and SpermGrad 100%; in these cases the operator can prepare the layers with whatever density is desired.

Moreover, some laboratories have used three layers [72]. When Percoll® was used, it was concluded that the three-layer discontinuous density gradient should be adopted as the preferred method for preparation of spermatozoa for assisted reproduction. Nowadays, however, the trend is for two layers to be used.

25.8.2 Swim-Up Technique

This technique is based on the principle that only the sperm with the best motility are able to rise to the supernatant [73]. This technique is the oldest sperm preparation method and was originally described by Mahadevan and Baker [74]. This procedure is very easy to perform and allows recovery of a good number of progressive spermatozoa in normozoospermic samples. However, it presents several disadvantages, given that it decreases the percentage of normally chromatin-condensed spermatozoa, while pelleted spermatozoa are in close contact with other cells, debris, and leukocytes at the bottom of the tube, being exposed to high levels of ROS [14].

This method is divided into the following steps:

1. Washing the semen sample with wash culture medium in a ratio of 1:2 and centrifuging the sample at 2200 rpm for 10 min.
2. Decanting the supernatant.
3. Adding new medium over the pellet, making it slide slowly over the walls of the tube to avoid mixing. The added volume depends on the quality of the sample and the size of the pellet (from 0.5 mL to 1.5 mL).
4. Placing the tube inside an incubator at 37 °C, inclined at an angle of 45°, for 45 min (this time may vary depending on the quality of the sample). During this step, progressive spermatozoa will migrate from the pellet to the upper fraction of the new culture medium.
5. When the upper fraction changes its color, this is the best moment to extract the sample from the incubator.

6. Recovering 0.5 mL of the upper fraction containing progressive spermatozoa.
7. Assessing the quality of the sample before it is used.

25.9 Processing of Retrograde Ejaculate Samples

A particular type of sperm preparation is needed to deal with cases of retrograde ejaculation, where spermatozoa must be retrieved from urine in the bladder. This condition is responsible for about 0.3–2% of infertility cases; thus, it is relatively infrequently encountered in practice [75].

The normal process of ejaculation requires compound coordination between the different parts of the male reproductive tract, including the epididymis, vas deferens, prostate, seminal vesicles, bladder neck, and bulbourethral glands, at least [76].

The normal process of ejaculation delivers sperm rapidly through the vas deferens and into the urethra by means of the ejaculatory ducts, traveling via the bladder neck thanks to contraction of the periurethral muscles. Then the sympathetic nervous system performs closure of the bladder neck and seminal emission is initiated.

Any factor that interferes with this tightly coordinated process may lead to retrograde passage of semen into the bladder; among them are pharmacological, neurogenic, or anatomical causes, including the use of pharmacological alpha-adrenergic blockers or psychotropic medications, neurogenic spinal cord injuries, lumbar sympathectomy, retroperitoneal lymph node dissection (RPLND), aortoiliac vascular surgery, abdominoperineal resection, diabetic autonomic neuropathy, multiple sclerosis, myelodysplasia, cerebrovascular accident, the presence of congenital posterior urethral valves, utricular cysts and exstrophy, acquired transurethral bladder neck incision, and transurethral resection of the prostate.

The way to proceed with these kinds of samples is initially focused on avoiding the harmful effects of urine—particularly its low pH—on sperm viability. This involves converting the urine to a neutral pH and then removing the spermatozoa as soon as possible after retrograde ejaculation. Second, to avoid difficulty in obtaining and processing additional samples if needed, sperm-freezing protocols are then applied.

The clinical protocol in this case starts with the patient taking bicarbonate (1–2 tablespoons dissolved in water) the night before the sample collection and then again on the morning of the sample collection, when urination to empty the bladder is recommended, followed by ejaculation, and then the next urine available is taken to the laboratory as soon as possible. In the laboratory, after measurement of the pH to check for the effect of bicarbonate, washing with sperm preparation medium is recommended, followed by centrifugation and elimination of the supernatant containing the urine where the spermatozoa were present. The pellet is then resuspended in clean culture medium and a search for motile spermatozoa is conducted to determine the sperm quality after the process. Finally, the sample is frozen if motile spermatozoa are found. In almost every case of retrograde ejaculation, this process guarantees availability of motile spermatozoa to be employed in ART.

25.10 Processing of Cryopreserved Ejaculate Samples

Although there is no literature regarding preferences when dealing with cryopreserved sperm preparation, sperm density gradients are the preferred choice in our experience. This affirmation is based on the rationale of the process itself in comparison with the swim-up.

Swim-up techniques require high-speed centrifugation to obtain well-formed pellets. With frozen–thawed samples, the large number of dead cells (due to the cryopreservation process itself) and cellular debris, together with the cryoprotectant compounds, lead to a sticky pellet, with increased difficulties for sperm to move through the culture medium, yielding lower recovery rates than density gradients. In this sense we refer to recovery rates as the percentage of total motile cells recovered, in comparison with the total motile cells available after thawing. It is interesting to note that the percentage of motile cells after density gradients with frozen–thawed sperm is significantly lower than that obtained with the swim-up method, but provided the total number of motile sperm available remains sufficient, as per the minimum threshold criteria—and in this sense, the density gradient method is superior to the swim-up method—clinical pregnancy rates are comparable.

25.11 Processing of Frozen Donor Samples

Further to the recommendations given in the previous section, the only aspect that is specific to the condition of working with donor sperm concerns the total amount of the sample that is thawed. In this case, the post-thawing tests (done on test vials) conducted the day on which the donor ejaculate is frozen are crucial, since their results will determine the sperm volume to be thawed.

With our particular sperm-freezing protocols, we are able to fine-tune the volume of the sperm sample to be thawed. We freeze sperm samples in approximately 50-microliter pills, enabling the possibility of deciding the total amount of the sample to be thawed, given that the frozen sample is aliquoted into 50-microliter units. In cases where the donor sperm is frozen in straws, the number of straws to be thawed will also depend on the expected survival rates and the total number of motile cells needed after thawing, as per the particular protocol that applies at each center.

The decision will be taken to obtain approximately 15–20 million total motile cells after the thawing process.

This will make sure that after sperm preparation by density gradients, at least two million total motile cells are available to be either inseminated via IUI or prepared for classical IVF or ICSI.

25.12 Troubleshooting in Sperm Processing

Sperm preparation by the swim-up and density gradient methods are well-established protocols, technically requiring little expertise and practical abilities.

Few negative or adverse circumstances are likely to arise in classical sperm preparation protocols. However, there is a possibility that the sample will have to be rejected if the sample traceability has been lost, the sample identification is misleading, the container is broken, or the sample is spilled.

Precautions should be taken especially at centers where batching of IVF is done, which is very common in developing countries. The quality of sperm preparation media imported from foreign countries may be compromised as a result of delays in transit and customs clearance. It is best to do a sperm survival study on each new lot of sperm-processing medium once it arrives at the clinic and before using it for any IVF batches.

25.13 Quality Control in Sperm Processing

The only quality control that can be exerted in sperm-processing protocols concerns the recovery rates. The typical mean recovery rates may remain around 15%. This means that out of the total number of motile cells in the ejaculate or after thawing, only 15% will be available after sperm preparation. Nevertheless, these thresholds must be established at each center so it can decide, on the basis of its own information and experience, what the mean recovery rates are and also what the acceptable fluctuations are. Then systems must be put in place to periodically (or, if possible, systematically, by means of real-time visualization systems) check for these means in sufficiently large series. In the event that a significant decrease is found, close supervision of protocol adherence by all staff is recommended.

Review Questions

1. What is the aim of sperm processing?
2. Why is it necessary to prepare sperm samples before using them in assisted reproductive technology?
3. Can sperm preparation be considered a diagnostic tool to select the most convenient assisted reproductive technology?
4. Which are the different parts of the ejaculate?
5. What are the reference values of the total motile sperm for each assisted reproductive technology?
6. Are consents and standard operating procedures important documentation in laboratories?
7. Are sperm-processing/selection methods the same in all types of samples or cases?
8. Is a post-thawing test recommended for frozen samples?

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PESA/MESA/TESA/TESE Sperm Processing

Sidney Verza Jr. and Sandro C. Esteves

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Learning Objectives

- PESA and TESA are simple and effective percutaneous surgical methods for retrieving epididymal and testicular spermatozoa, respectively, in men with obstructive azoospermia.
- MESA is an efficient microsurgical method to retrieve sperm from the epididymis of men with obstructive azoospermia. MESA has the advantage of achieving higher numbers of sperm aspirated than PESA, favouring cryopreservation and allowing multiple ICSI attempts without the need of repeat retrievals.
- TESE, preferentially using microsurgery (micro-TESE), is the preferred approach to retrieve sperm from men with non-obstructive azoospermia. Micro-TESE improves sperm extraction efficacy and efficiency, as higher sperm retrieval rates and lower tissue removal are achieved than conventional sperm retrieval methods. The latter facilitates laboratory sperm processing.
- The primary goal of PESA/MESA/TESA/TESE sperm processing is the recovery of a clean sample containing motile sperm. Since these specimens are more fragile—and often compromised in quality—compared to ejaculated counterparts, laboratory techniques should be carried out with great care to avoid jeopardizing sperm fertilizing potential. The whole process starts with the surgical collection of the best-quality specimen as possible. During laboratory steps, minimal iatrogenic cellular damage may be achieved by strict control of centrifugation force and duration, exposure to ultraviolet light and temperature variation, laboratory air quality conditions, as well as the use of high-quality reagents, materials, and equipment.
- PESA and MESA sperm processing can be performed either by mini-gradient centrifugation or simple washing using small volumes of culture media.
- TESA and TESE specimens are processed by mechanical mincing of seminiferous tubules, followed by enzymatic digestion if required.
- The use of immotile epididymal/testicular sperm for ICSI negatively impacts clinical outcomes. Methods for selecting viable immotile sperm for ICSI include the HOST, STFT, and motility stimulants.
- Cryopreservation is used to intentionally freeze retrieved sperm for future use or surplus specimens that would be discharged after ICSI. Novel methods have been introduced to freeze low sperm number and quality with promising results.

26.1 Introduction

The development of intracytoplasmic sperm injection (ICSI) was a breakthrough for men with severe male factor infertility [1]. Equally important was the application of ICSI to azoospermic men and the confirmation that spermatozoa taken from either the epididymis or testis were capable of normal fertilization and pregnancy [2, 3].

Azoospermia is clinically divided into obstructive and non-obstructive. Obstructive azoospermia (OA) is characterized by normal spermatogenesis in which either a mechanical blockage exists somewhere between the rete testis and ejaculatory duct or the epididymis and vas deferens are absent (total or partial agenesis). Acquired OA is due to vasectomy; failure of vasectomy reversal; post-infectious diseases; surgical procedures in the scrotal, inguinal, pelvic, or abdominal regions; and trauma. Congenital OA is due to congenital absence of the vas deferens (CAVD), ejaculatory duct or prostatic cysts, and Young's syndrome [4].

Unlike OA, non-obstructive azoospermia (NOA) comprises a spectrum of severe testicular disorders resulting from various causes that include endocrine and genetic abnormalities, post-infectious diseases, trauma, environmental toxins, exposure to gonadotoxins (radiation, chemotherapy), cryptorchidism, varicocele; however, the condition may also be idiopathic. Whereas it is relatively easy to harvest sperm in men with OA, NOA represents a challenge for sperm retrieval [4, 5].

Several methods have been developed to retrieve epididymal and testicular sperm from azoospermic men. Among them, percutaneous epididymal sperm aspiration (PESA) [6] and microsurgical epididymal sperm aspiration (MESA) [2] are the techniques used to retrieve sperm from the epididymis, whereas testicular sperm aspiration (TESA) and testicular sperm extraction (TESE) are the methods to harvest sperm from the testicle in OA [6, 7]. In contrast, open testicular sperm extraction (TESE) using single or multiple biopsies [8–10] and TESE with the aid of microsurgery (microdissection testicular sperm extraction, micro-TESE) are methods to harvest sperm from men with NOA [11–13].

Processing surgically retrieved spermatozoa differs from the commonly used methods for processing ejaculates. In the former, sperm processing should not only ease the selection of the best-quality spermatozoa for ICSI but also optimize their fertilizing ability, whenever possible. The laboratory has a crucial role in handling the often-compromised surgically retrieved samples, particularly in NOA and cryopreserved specimens. Lab personnel should receive the best-quality extracted sample as possible, with minimal or no contaminants such as red blood cells and harmful microorganisms. Furthermore, mastering the processing techniques minimizes iatrogenic cellular damage during sperm processing. Among the methods used to properly handle such specimens, controlling centrifugation force and duration, exposure to ultraviolet light and temperature variation, laboratory air quality conditions, dilution and washing steps, quality of reagents, culture media, and disposable materials are of utmost importance. Lastly, if possible, techniques to improve sperm fertilizing potential should be applied, including the use of stimulants and methods to select viable sperm for ICSI. The latter is particularly important when only immotile spermatozoa are available.

In this chapter, our goals are to provide a step-by-step laboratory description of the commonly used methods for PESA/MESA/TESA/TESE sperm processing. Also, we provide protocols for identifying viable immotile sperm for

ICSI. We included our published data about the use of testicular and epididymal sperm for ICSI in azoospermic men and, lastly, provided valuable tips for processing such extracted sperm.

26.2 Step-by-Step Protocol

26.2.1 Materials, Equipment, Reagents, and IVF Laboratory Setup

26.2.1.1 Operating Room

- Sterile surgical gloves and syringes (1 mL, 20 mL)
- 0.7 × 25 mm, 0.45 × 13 mm, 1.2 × 40 mm (TESA/PESA) disposable needles
- 2% lidocaine
- Heating block for test tubes
- Syringe holder (TESA; see Fig. 26.3)
- Surgical loupe (PESA and TESA)
- Operating microscope (micro-TESE and MESA)
- Microsurgery instruments (micro-TESE and MESA)

26.2.1.2 IVF Laboratory

- Petri dishes
- Disposable serological pipettes
- Pipettor 1–200 μL and sterile tips
- Pipetting device
- 6-mL sterile centrifuge polystyrene tubes with caps
- 0.7 × 25-mm needles and tuberculin syringes
- Marker pen
- ICSI micropipettes
- Laminar flow cabinet
- Warming plate
- Stereomicroscope
- Centrifuge
- Inverted microscope equipped with Hoffman modulation contrast and electro-hydraulic micromanipulators
- HEPES-buffered Human Tubal Fluid (HTF) and Human Serum Albumin (HSA)
- Mineral oil
- Polyvinylpyrrolidone (PVP) solution
- Colloidal density gradient
- Erythrocyte lysing buffer
- Collagenase
- Pentoxifylline solution

26.2.1.3 Laboratory Setup

Note: Sterile handling conditions under a laminar flow cabinet or clean room environment should be used during all laboratory steps.

- Prepare a 10-mL (for PESA) or 20-mL (TESA/TESE/MESA) HEPES-buffered protein-supplemented (5% HSA) sperm culture medium, and keep it at 37 °C.
- Transfer a 5-mL aliquot of sperm culture medium to a 6-mL polystyrene tube, and send it to the operating room (buffered sperm media is used to flush the syringe + needle before aspiration and to incubate epididymal aspirates or testicular specimens).
- Place two Petri dishes on a warm surface (37 °C) inside the workstation (for PESA only).
- Prepare 4-well dishes (e.g. Nunc) by adding 0.5-mL sperm medium aliquots to each well (TESE). Keep them onto a warm surface (37 °C) inside the workstation.
- Mount two tuberculin syringes connected with 13-gauge (to be used as tools for mincing and squeezing seminiferous tubules in TESA/TESE processing).

26.2.2 PESA and MESA Sperm Processing

26.2.2.1 Surgical Technique

Note: We perform PESA and MESA under local and intravenous anaesthesia [14, 15].

For PESA

- A 10-mL solution of 2% lidocaine is injected around the spermatic cord near the external inguinal ring upon patient unconsciousness is achieved. The epididymis is stabilized between the index finger, thumb, and forefinger, while the testis is held with the palm of the hand.
- A 23-gauge needle attached to a 1-mL tuberculin syringe is inserted into the epididymis through the scrotal skin. Loupe magnification is used to avoid injuring small vessels seen through the skin (Fig. 26.1).
- Negative pressure is created, and the tip of the needle is gently moved in and out within the epididymis until fluid enters the syringe. The amount of epididymal fluid obtained during aspiration is often minimal (~0.1 mL), except in cases of CAVD in which more fluid can be aspirated.
- The needle is withdrawn from the epididymis, and the aspirate is flushed into a 0.5-mL 37 °C sperm medium.
- The tube containing the epididymal aspirate is transferred to the IVF lab. PESA is repeated at a different site of the same epididymis (from cauda to caput) or the contralateral one until an adequate number of motile sperm are retrieved. If PESA fails to retrieve motile sperm for ICSI, TESA is performed at the same operative time (Fig. 26.1).

For MESA

Note: Operating microscope and microsurgery technique are used throughout the procedure, as previously described [14, 16] (Fig. 26.2).

- A 10-mL solution of 2% lidocaine is injected around the spermatic cord near the external inguinal ring upon patient unconsciousness is achieved. After anaesthetic blockade of the spermatic cord, the anterior scrotal skin is stretched, and the skin and tunica vaginalis are infiltrated with 2 mL of 2% lidocaine. A transverse 3-cm incision is made through the anaesthetized layers, and the testis is exteriorized.

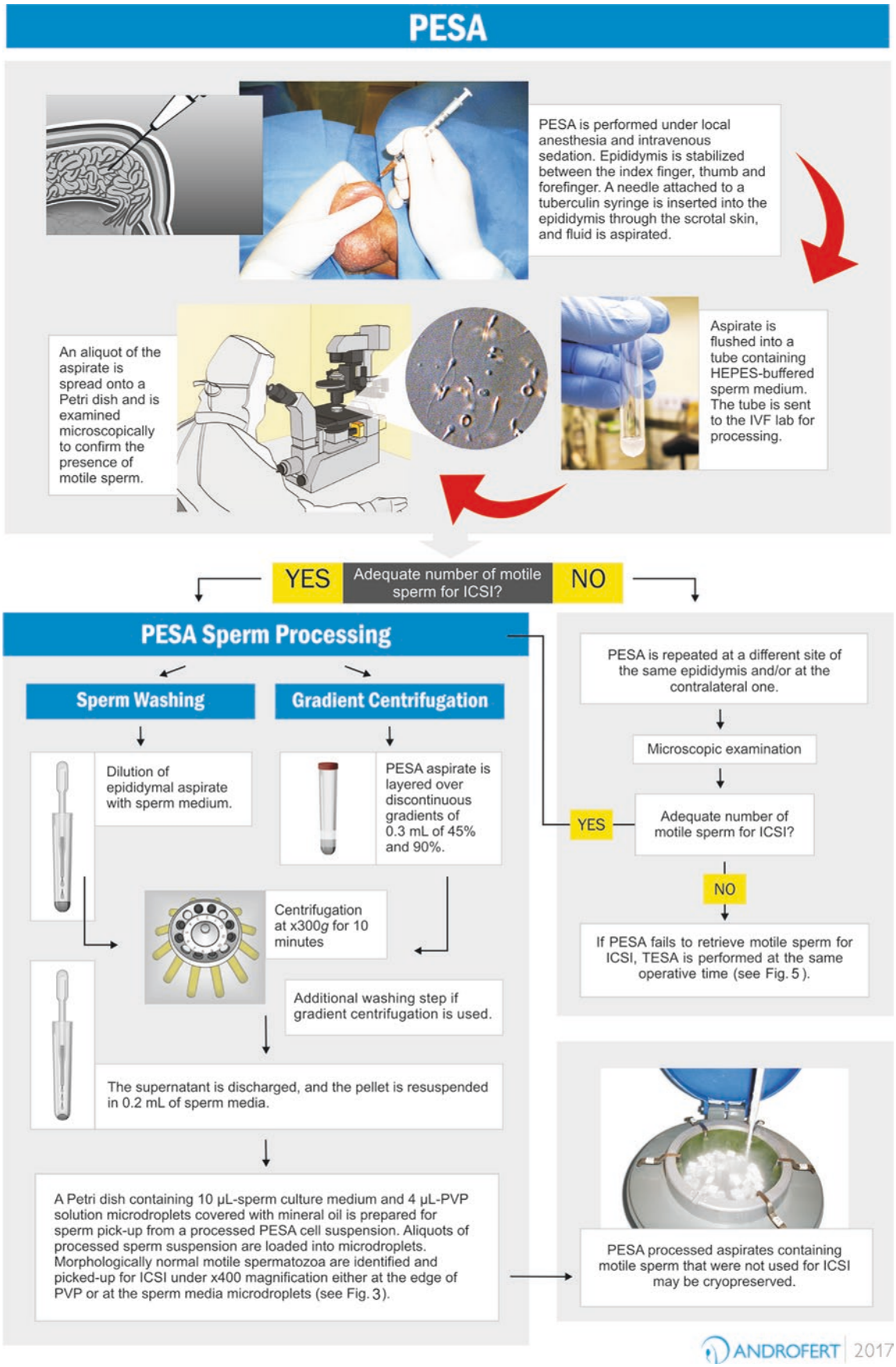

Fig. 26.1 PESA sperm processing. The flow chart illustrates the PESA steps from surgical procedure to processing of epididymal aspirates for ICSI or cryopreservation

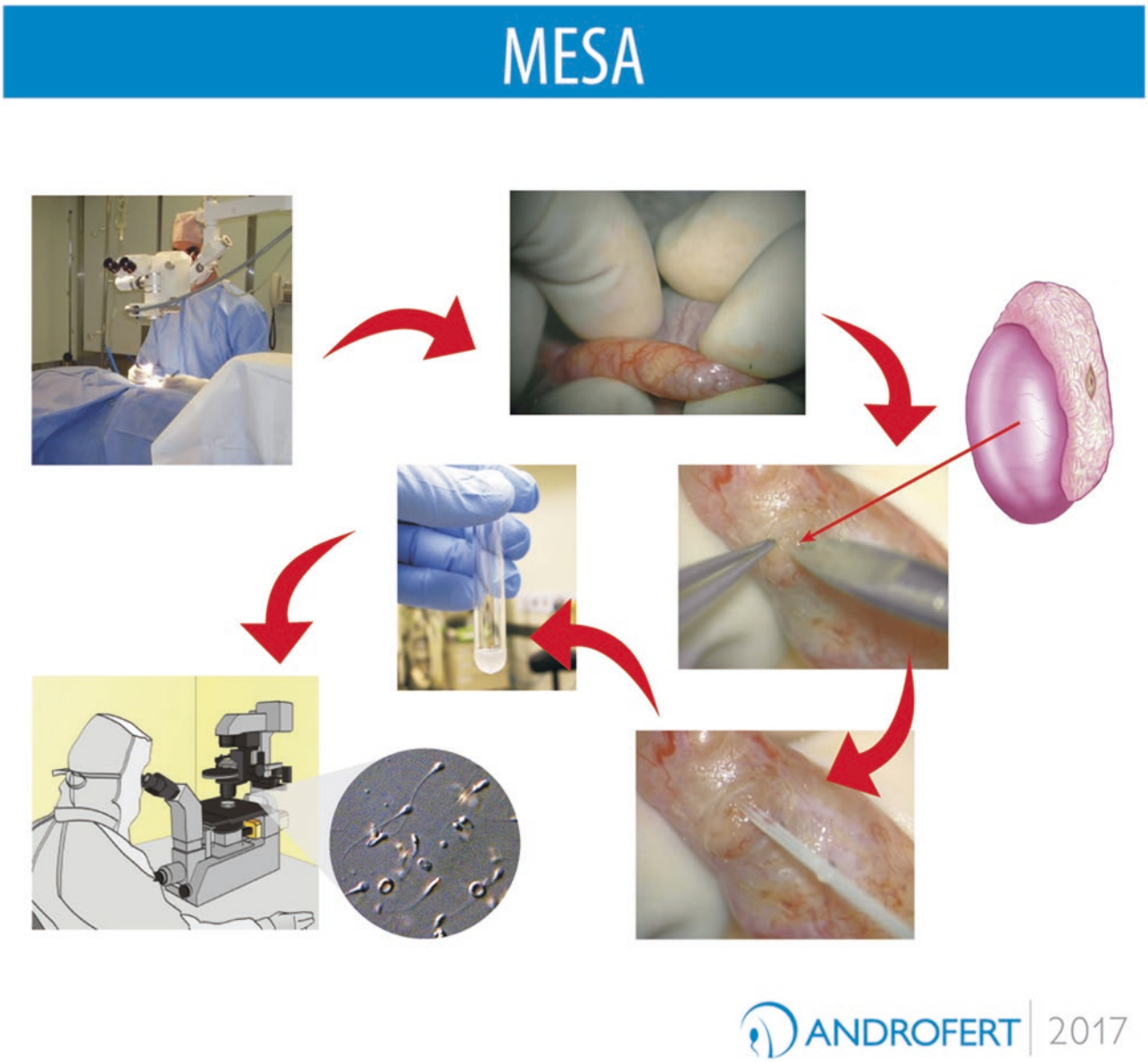

Fig. 26.2 Microsurgical epididymal sperm aspiration (MESA). After exposure of the testis and epididymis, a dilated epididymal tubule is dissected and opened. The fluid is aspirated, diluted with sperm medium, and sent to the laboratory for examination

- The epididymal tunica is incised, and an enlarged tubule is selected. An epididymal tubule is dissected and opened with sharp microsurgical scissors. The fluid that flows out of the tubule is aspirated with the aid of a silicone tube or a needle attached to a tuberculin syringe (Fig. 26.2).
- The aspirate is flushed into a tube containing warm sperm medium and transferred to the laboratory for examination. MESA can be repeated at a different site on the same epididymis (from the cauda to caput regions) or the contralateral epididymis until an adequate number of motile sperm are retrieved. If MESA fails to retrieve motile sperm, TESA or TESE can be performed as part of the same procedure. However, MESA often provides enough sperm for cryopreservation. A single MESA usually enables the retrieval of a large number of high-quality sperm that can be used for ICSI or intentionally cryopreserved for subsequent ICSI attempts.

26.2.2.2 Laboratory Handling of Epididymal Aspirates

- Identify aspirate-containing tube(s) according to the epididymis side and site of aspiration (caput, body, or tail). Homogenize the epididymal aspirate to avoid sperm agglutination (epididymal spermatozoa tend to agglutinate fast). Keep aspirate-containing tubes capped at 37 °C.

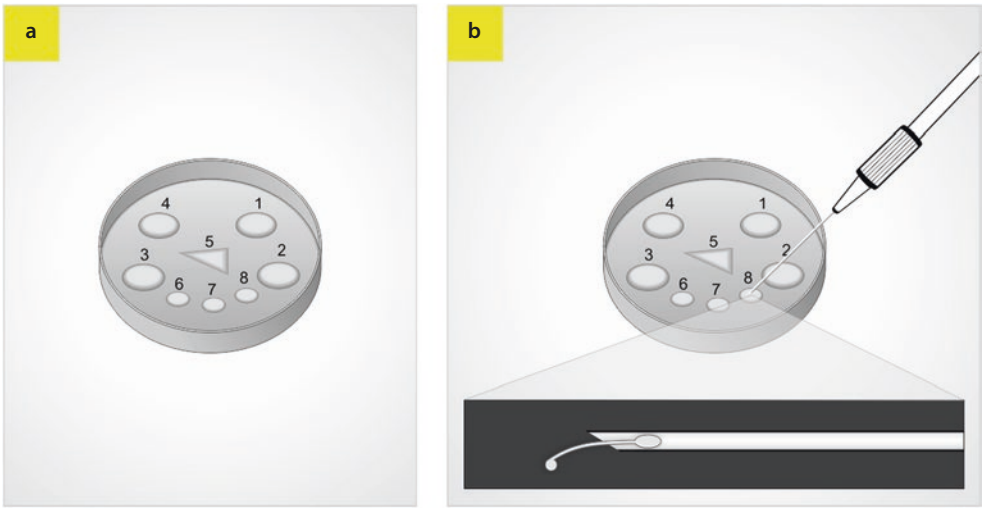

Fig. 26.3 Preparation of Petri dishes. A 50 × 09-mm Petri dish containing several microdroplets of culture medium under mineral oil is prepared for sperm pick-up from a processed epididymal or testicular cell suspension. Microdroplets are prepared as follows: four 10-μL sperm medium at dish periphery to load specimens (numbered 1–4), one 4-μL polyvinylpyrrolidone (PVP) at dish centre in a triangular shape to pick up selected sperm for ICSI (number 5), and two to three 10-μL sperm medium at dish centre below the PVP triangle for washing (numbered 6–8). Alternatively, one of the sperm medium-containing microdroplets (e.g. number 8) or the outer ones (1–4) is replaced with the hyposmotic or motility stimulant solutions, respectively **a**. The hyposmotic swelling test is illustrated **b**. The sperm tail is partially withdrawn from the injection micropipette into the HOS droplet. A swelling at the level of the tail tip may be seen under the inverted microscope with contrast at ×400 magnification

- Place a 10–20-μL specimen aliquot onto a Petri dish, and spread it using a micropipette tip. Examine the fluid under the inverted microscope to confirm the presence of motile sperm. Inform the surgeon promptly if an adequate number of motile sperm for ICSI are available. If more PESA specimens are taken, pool samples of similar quality together for processing or cryopreservation. If TESA specimens are obtained, process specimens according to the "TESA processing protocol" (Fig. 26.5).
- Identify aspirate-containing tube(s) according to the presence of motile sperm. Make a decision regarding the processing method to be used, i.e., simple washing or two-layer discontinuous mini-gradient centrifugation, based on a gross estimate of sperm count and motility. We use gradient centrifugation when the sperm number is high, particularly if contaminated with red blood cells, cellular debris, and immotile sperm. Otherwise, we use a simple washing.
- For density gradient centrifugation, layer an aliquot of the PESA aspirate up to 0.5-mL over 0.3-mL gradients of 45% and 90%, respectively, and centrifuge at ×300 g for 10 min. Resuspend the pellet in 1.5-mL fresh sperm medium and repeat centrifugation. Remove the supernatant carefully, leaving about 0.2 mL of the medium above the pellet. Resuspend the pellet and keep it at 37 °C until use or cryopreservation.
- For simple washing, dilute the epididymal aspirate with fresh sperm medium to a final volume of 1.5–2.0 mL. Centrifuge the mixture at ×300 g for 10 min, discharge the supernatant, and resuspend the pellet in 0.2-mL sperm medium. If the processed PESA specimen is still contaminated with an excessive number of red blood cells, make a new dilution with 2-mL erythrocyte lysing buffer and centrifuge the mixture (Appendix).
- Prepare a Petri dish containing a series of microdroplets under mineral oil for sperm pick-up from a processed epididymal cell suspension (Fig. 26.3).
- Load a 1-μL sperm suspension aliquot gently into the centre of the PVP droplet if the specimen contains motile sperm with progressive motility. Morphologically normal motile spermatozoa can be identified and picked up for ICSI with the injection micropipette at the edge of the PVP droplet. If progressive motility is low or absent and/or the specimen is contaminated with cellular debris, load a sperm suspension aliquot (1–4 μL) into each 10-μL outer microdroplet of HEPES-buffered culture medium to facilitate search and selection of motile sperm.
- Aspirate a small volume of PVP into the injection micropipette to improve control during sperm pick-up and to avoid blowing air bubbles during ejection of selected sperm into the PVP droplet.
- Pick up morphologically normal and motile and place them close to the edge of PVP. Make a final morphologic sperm assessment under ×800 magnification in the group of preselected spermatozoa for ICSI. Immobilize and inject the selected spermatozoa into the cytoplasm of metaphase II oocytes.

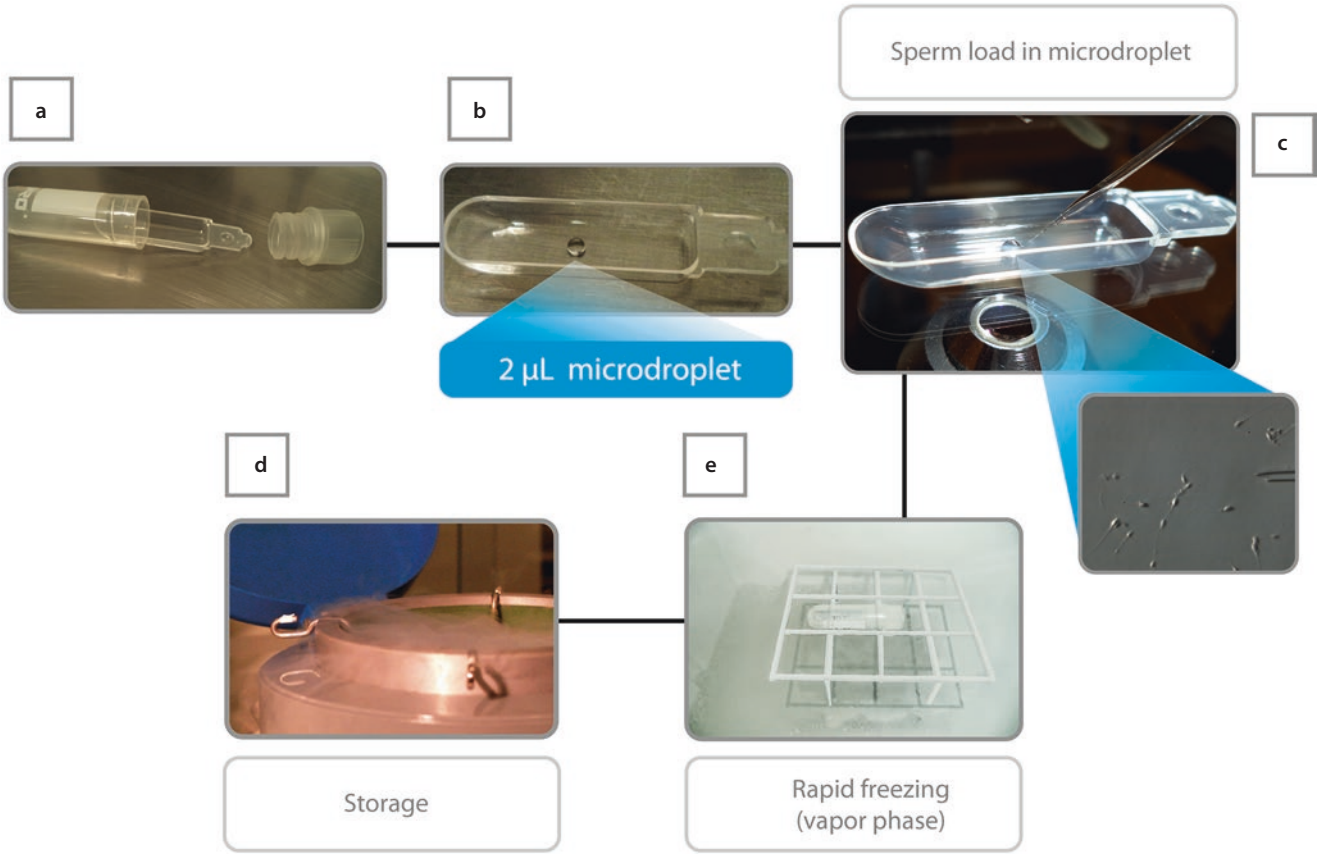

Fig. 26.4 The cell sleeper method for low count sperm freezing. The Cell Sleeper (Nipro, Japan) consists of an outer vial, an inner tray, and screw cap **a**. The inner tray is removed from the vial and placed in the lid of a large culture dish and a 2-µL droplet of cryopreservation solution is pipetted into the tray, in a central position **b**. Spermatozoa are aspirated and ejected into the droplet on the tray with the aid of a microinjection pipette **c**. Immediately thereafter, the tray is returned to the vial and the vial is closed with the screw cap. The vial is placed in a horizontal position 4–5 cm above the surface of liquid nitrogen **d**. After 2 min, the vial is submerged in LN2 and secured into a cryopreservation cane for storage **e**. (Reprint with permission from Springer, Esteves [20])

- Cryopreserve surplus PESA processed aspirates containing motile sperm not used for ICSI (or total specimen if intentional cryopreservation). We freeze PESA specimens using either (i) fast liquid nitrogen vapour method [17] or (ii) cell sleeper method [18, 19] or both. Cell sleepers are nonbiological closed devices in which a few spermatozoa can be frozen in microdroplets (Fig. 26.4). The method involves rapid freezing and has shown to be reliable for freezing surgically retrieved testicular sperm [18, 19].

26.2.3 TESA Sperm Processing

26.2.3.1 Surgical Technique

Note: We perform TESA under local and intravenous anaesthesia [14], as described elsewhere.

- After anaesthetic blockade of the spermatic cord, the epididymis is stabilized between the index finger, thumb, and forefinger while the anterior scrotal skin is stretched.
- A 13-gauge needle attached to a 20-mL syringe is connected to a syringe holder and is inserted through the stretched scrotal skin into the anteromedial or anterolateral portion of the superior testicular pole, at an oblique angle towards the medium and lower poles (Fig. 26.5). Loupe magnification is used to avoid small vessels seen through the skin.
- Negative pressure is created by pulling the syringe holder, whereas the needle is moved in and out within the testis in an oblique plane to disrupt the seminiferous tubules and sample different areas. When a small piece of testicular tissue is aspirated, the needle is gently withdrawn from the testis while the negative pressure is maintained. A pair of microsurgery forceps is used to grab the seminiferous tubules that exteriorize from the scrotal skin, thus aiding in the removal of the specimen (Fig. 26.5).
- The specimen is flushed into a tube containing 0.5–1.0-mL warm sperm medium and is transferred to the IVF lab. TESA can be performed at the contralateral testis at

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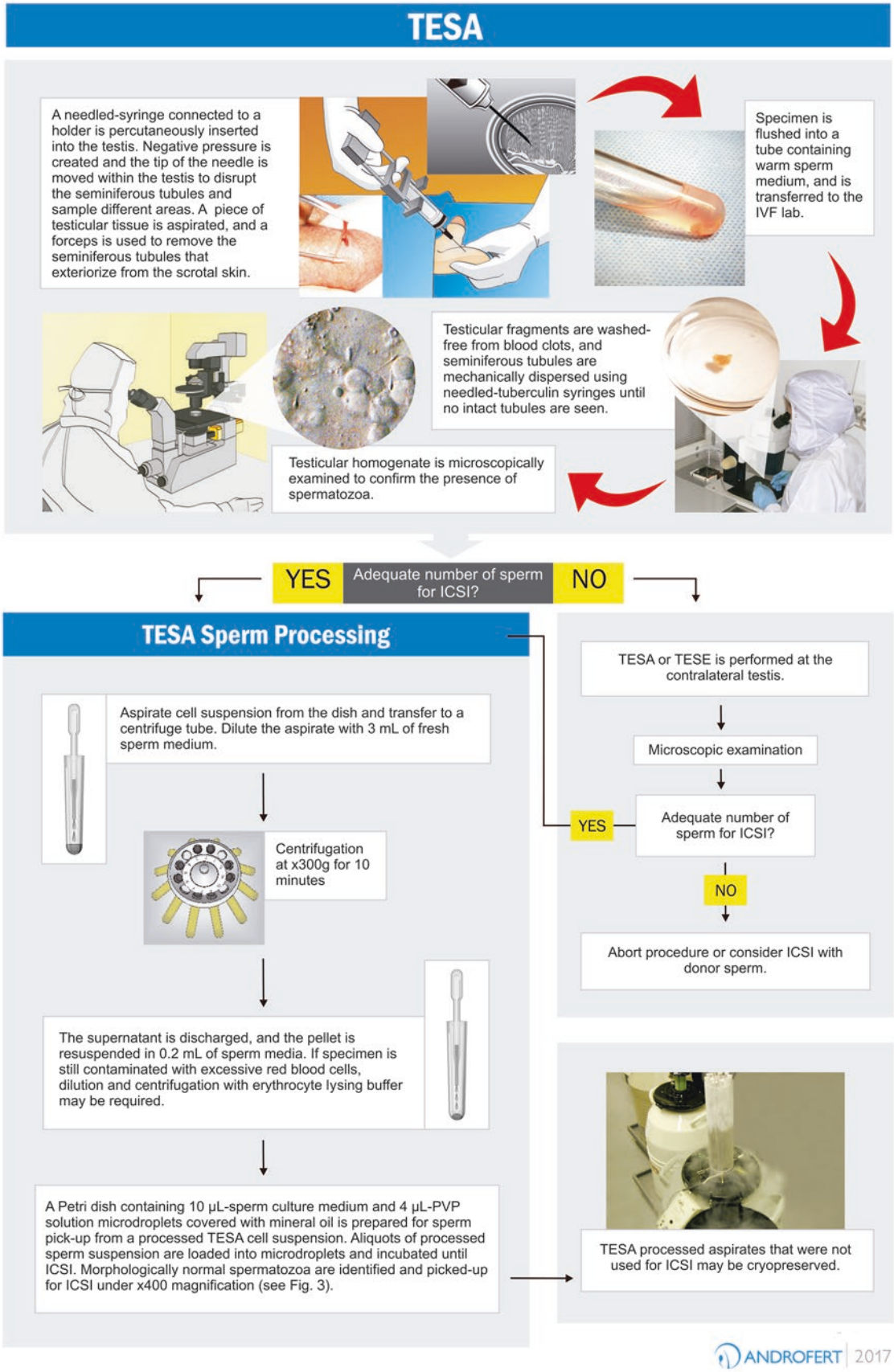

Fig. 26.5 TESA sperm processing. Flow chart illustrates TESA steps from the surgical procedure to the processing of testicular specimens for ICSI

the same operative time if an insufficient number of spermatozoa are retrieved for ICSI.

26.2.3.2 Laboratory Handling of Testicular Aspirates

- Discharge the TESA aspirate onto the Petri dish. Under the stereomicroscope, identify the seminiferous tubules and remove blood clots using the needled-tuberculin syringes.
- Transfer the seminiferous tubules to a Petri dish containing fresh sperm medium. Perform mechanical dispersion by mincing the tubules using both needled-tuberculin syringes (use one to hold tubules in place at the bottom of the dish and the other to squeeze and open them). Repeat this step until no intact tubules are seen.
- Examine the homogenate to confirm the presence of sperm using the inverted microscope at ×400 magnification. Inform the surgeon promptly if an adequate number of spermatozoa for ICSI are available. If other TESA specimens are taken, carry out the initial processing steps as described above.
- Aspirate and transfer the cell suspension to a sterile centrifuge tube. Dilute the aspirate with 3 mL of fresh sperm medium and wash it at ×300 g for 7 min. Discharge the supernatant and resuspend the pellet in 0.2 mL of sperm medium. If the processed TESA specimen is contaminated with an excessive number of red blood cells, dilute the specimen with erythrocyte lysing buffer and perform an additional centrifugation step (Appendix).
- Prepare Petri dishes as described in the "PESA protocol" for sperm pick-up (Fig. 26.3). Load 1–2-μL sperm suspension aliquot at each 10-μL outer microdroplet of HEPES-buffered culture medium to facilitate sperm search and pick-up.
- Pick up morphologically normal and motile and place them close to the edge of PVP. Make a final morphologic sperm assessment under ×800 magnification in the group of preselected spermatozoa for ICSI. Immobilize and inject the selected spermatozoa into the cytoplasm of metaphase II oocytes.
- Cryopreserve surplus TESA processed aspirates containing motile sperm not used for ICSI (or entire specimen if intentional cryopreservation). We freeze TESA specimens using either (i) fast liquid nitrogen vapour method [17] or (ii) cell sleeper method [18, 19] or both.

26.2.4 TESE Sperm Processing

26.2.4.1 Micro-TESE Surgical Technique

Note: We perform micro-TESE under local and intravenous anaesthesia [14], as described elsewhere. For micro-TESE, operating microscope and microsurgery technique are used throughout the procedure, as previously described [16] (Fig. 26.6).

- After anaesthetic blockade of the spermatic cord, the anterior scrotal skin is stretched, and the skin and tunica vaginalis are infiltrated with 2 mL of 2% lidocaine. A transverse 2-cm incision is made through the anaesthetized layers, and the testis is exteriorized.
- A single, large, mid-portion incision is made in an avascular area of the tunica albuginea under ×6–8 magnification, and the testicular parenchyma is widely exposed.
- Dissection of the testicular parenchyma is carried out at ×16–25 magnification searching for enlarged seminiferous tubules, which are more likely to contain germ cells and normal sperm production (Fig. 26.7). The superficial and deep testicular regions may be examined, if necessary, and microsurgery-guided testicular biopsies are performed by removing the enlarged tubules (Fig. 26.6). If enlarged tubules are not seen, then any tubule different than the remaining ones in size is excised [11]. If all tubules are identical in appearance, random micro-biopsies (at least three at each testicular pole) are performed.
- Each excised testicular tissue specimen is placed in a Petri dish containing sperm media. Specimens are washed grossly to remove blood clots and are sent to the IVF laboratory for processing.

26.2.4.2 Laboratory Handling of Testicular Specimens

- Under the stereomicroscope, remove blood clots using the needled-tuberculin syringes. Transfer fragments to another dish well containing fresh medium and wash again until no blood clots are seen.
- Perform mechanical dispersion by mincing the tubules using both needled-tuberculin syringes (Fig. 26.5). In our setup, a minimum of two laboratory technicians/embryologists work together to speed up the sperm searching process (one mincing the tubules under the stereomicroscope and the other searching for spermatozoa under the inverted microscope).
- Examine the homogenate to confirm the presence of sperm using the inverted microscope at ×400 magnification. The surgeon should be informed promptly if any sperm is found to allow him to decide on continuing micro-TESE in the same testis or moving to the contralateral side. If other TESE specimens are taken, carry out the initial processing steps as described above.
- Aspirate and transfer the cell suspension to a sterile centrifuge tube. Dilute the aspirate with 3 mL of fresh sperm medium and wash it at ×300 g for 7 min. Discharge the supernatant and resuspend the pellet in 0.2 mL of sperm medium. If the processed TESE specimen is contaminated with an excessive number of red blood cells, dilute the specimen with erythrocyte lysing buffer and perform an additional centrifugation step (Appendix).

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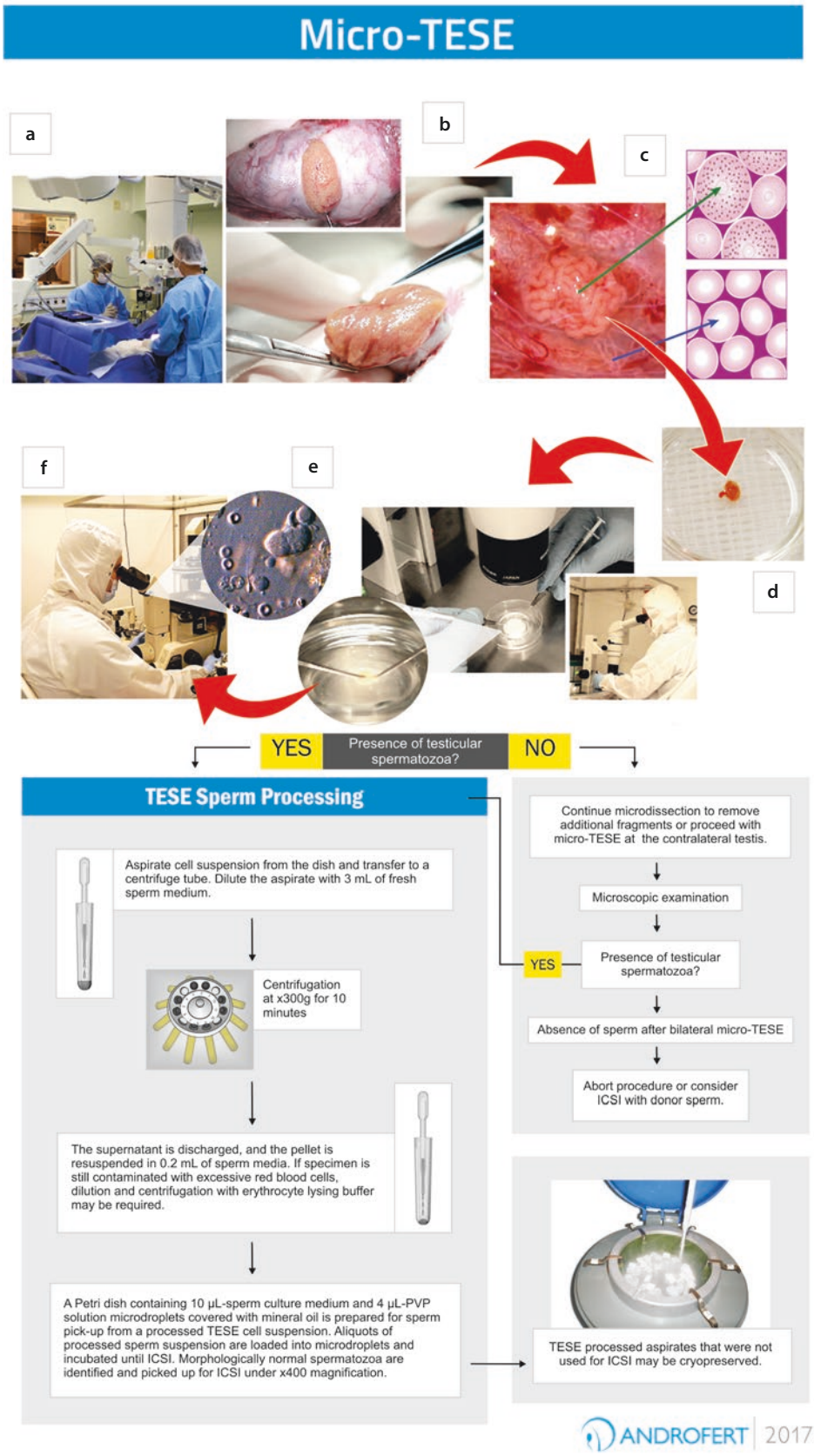

Fig. 26.6 TESE sperm processing. The flow chart illustrates the micro-TESE steps from the surgical procedure to processing of testicular specimens for ICSI. The rationale of micro-TESE is to identify focal areas of sperm production within the testes, based on the size and appearance of the seminiferous tubules, with the aid of the operating microscope **a**. A large incision is made in an avascular area of the tunica albuginea, and the testicular parenchyma is widely exposed **b**. The parenchyma is then dissected at ×16 to ×25 magnification to enable the search and isolation of seminiferous tubules exhibiting larger diameter in comparison with nonenlarged or collapsed counterparts **c**. These enlarged tubules are more likely to contain germ cells and eventually normal sperm production. Microsurgery-guided biopsies are performed by carefully removing such tubules, which are sent to the laboratory for examination **d**. The minimal tissue extracted facilitates laboratory processing and sperm search, thus increasing the process efficiency **e**. The initial laboratory step involves mechanical mincing of the seminiferous tubules and examination of specimens for sperm identification **f**. The use of optical magnification also reduces the chances of vascular injury by proper identification of testicular blood supply, thus reducing the chances of haematoma formation and testicular devascularization

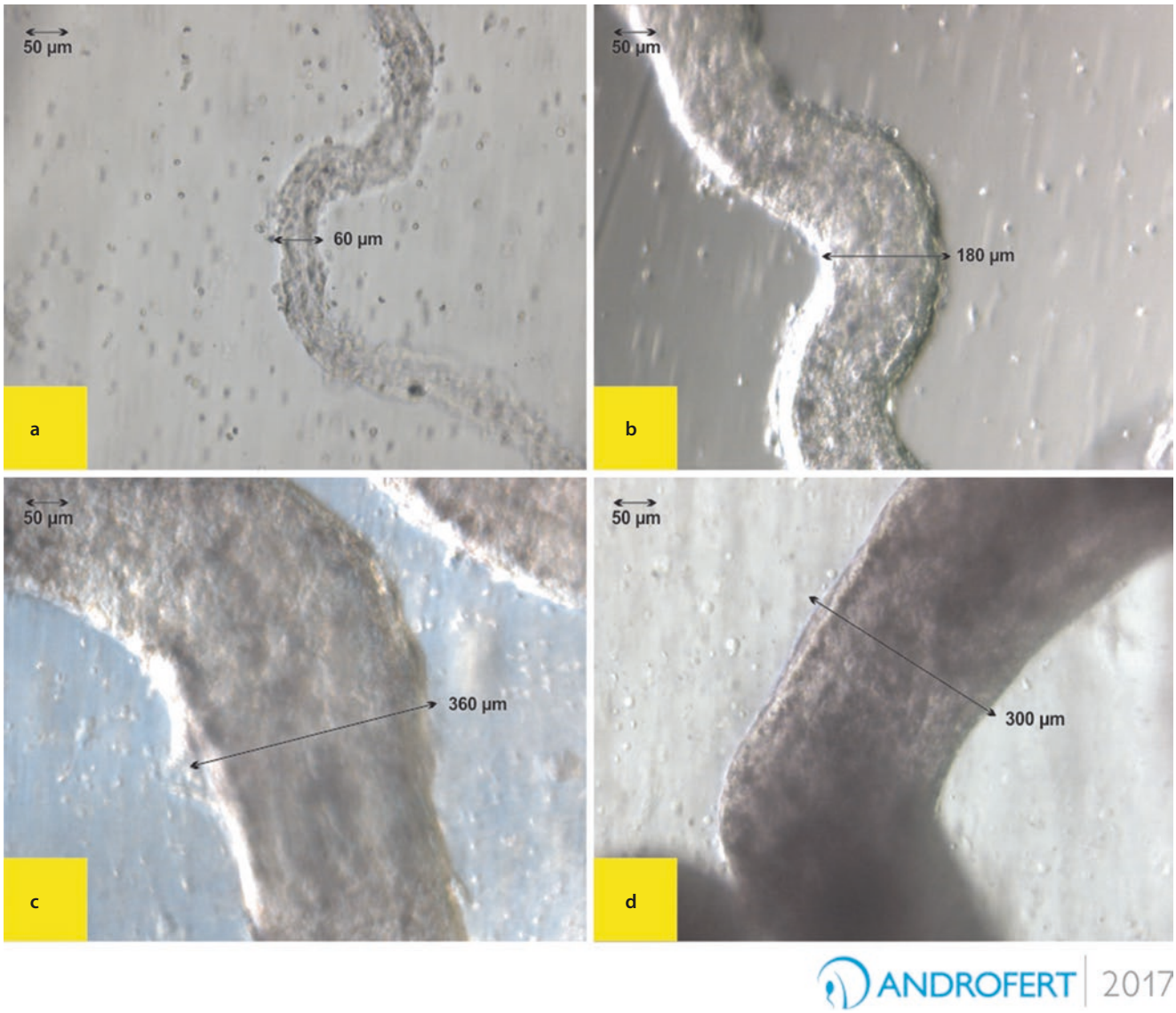

Fig. 26.7 Preprocessing microscopic appearance of fresh seminiferous tubules (Magnification: 100×, inverted optical microscope, Nikon Eclipse Diaphot 300 with phase contrast (Hoffman)). **a** and **b** Thin seminiferous tubules, as usually observed in the cases of Sertoli cell only; **c** and **d** seminiferous tubules of larger diameter than the previous ones **a** and **b**, compatible with the presence of germ cells and mature sperm

- Prepare Petri dishes as described in the "PESA protocol" for sperm pick-up (Fig. 26.3). Load 1–2-µL sperm suspension aliquot at each 10-µL outer microdroplet of HEPES-buffered culture medium to facilitate sperm search and pick-up.
- Pick up morphologically normal and motile and place them close to the edge of PVP. Make a final morphologic sperm assessment under ×800 magnification in the group of preselected spermatozoa for ICSI. Immobilize and inject the selected spermatozoa into the cytoplasm of metaphase II oocytes.
- Cryopreserve surplus TESE processed aspirates containing motile sperm not used for ICSI (or entire specimen if intentional cryopreservation). We freeze TESE specimens using the cell sleeper method (Fig. 26.4).
- Perform enzymatic digestion of testicular specimens with collagenase and repeat the search if no spermatozoa are found on both the initial and extended search (Appendix).

26.2.5 Laboratory Handling of Cryo-thawed Epididymal and Testicular Specimens

26.2.5.1 Cryopreservation by Fast Liquid Nitrogen Vapour Method

After thawing, removal of cryoprotectant is carried out by simple washing [21], as described in the "TESA sperm processing protocol".

26.2.5.2 Cryopreservation by "Cell Sleeper" Method

- Prepare a Petri dish as follows (warm stage off): four microdroplets containing each 10 μL of culture medium, two microdroplets containing 10 μL of culture medium and pentoxifylline [22] (final concentration 5 mM; see Appendix), one microdroplet containing 1 μL of culture medium, and one microdroplet containing 4.5 μL of PVP in a triangular shape (Fig. 26.3). Cover with mineral oil and warm the dish at 37 °C for 20 min.
- Prepare the micromanipulator using a micropipette with a large inner diameter (e.g. 5.5 μm) to facilitate capture of spermatozoa.
- Remove the cryovial from the liquid nitrogen cylinder and leave it capped at room temperature for 1 min.
- Open the cryovial and remove the inner tray. Using a micropipette, remove approximately 50–80% of the cell sleeper droplet volume and transfer it directly to one of the culture medium droplets of the Petri dish.
- Cover the cell sleeper platform with pre-warmed (37 °C) 300 μL of mineral oil.
- Place the cell sleeper tray onto the inverted microscope stage, lower the injection needle into the cryoprotectant drop where the sample is, and start to search for spermatozoa.
- Aspirate sperm into the injection needle, raise the needle, withdraw the tray, and insert the previously prepared Petri dish onto the inverted microscope.
- Leave the specimens in the culture medium droplets for at least 15 min to allow diffusion of cryoprotectant and cell hydration.
- Transfer the cells as follows: motile spermatozoa are transferred to PVP; immotile spermatozoa are transferred to the droplet containing pentoxifylline. Utilize a sperm selection method (see section below) if only immotile sperm is seen after pentoxifylline incubation.
- Perform sperm immobilization and microinjection as routinely done using motile or immotile sperm.

26.2.6 Methods for Selecting Viable Immotile Sperm for ICSI

Conventional seminal parameters have little or no influence in ICSI outcomes, except when only immotile spermatozoa are available [23, 24]. In such cases, fertilization rates are reduced. Different strategies may be used to differentiate live immotile spermatozoa from dead ones, thus aiding in the selection of viable gametes for ICSI, as described below.

26.2.6.1 Hyposmotic Swelling Test (HOST) [25–27]

- Using the microinjection pipette, pick up morphologically normal immotile spermatozoa from the sperm medium droplet and transfer to PVP.
- Aspirate a single spermatozoon head first into the pipette.
- Move the pipette into the HOS microdroplet (Fig. 26.3 and Appendix) and release only the sperm tail into the HOS solution. Keep it for 5–10 s and observe if a tail tip swelling occurs (sperm tail swelling is often minimal and is a marker of viability in fresh specimens, but may not be suitable for testing cryopreserved ones [27]).
- If tail swelling is seen, aspirate the cell back to the pipette and release it in a drop of fresh medium to allow osmotic re-equilibration (tail swelling often disappears in 5–20 s). If tail swelling is not seen, discharge spermatozoon into the HOS solution.
- Transfer the viable selected spermatozoon to the PVP drop. Repeat these steps until sufficient number of viable sperm is selected for ICSI.

26.2.6.2 Sperm Tail Flexibility Test (STFT) [28, 29]

- Using the microinjection pipette, pick up morphologically normal immotile spermatozoa from sperm microdroplet (Fig. 26.3) and transfer to the PVP solution.
- Align the spermatozoa near the PVP droplet edge.
- Touch sperm tail with the tip of the microinjection pipette, and force the tail to move up and down. Tail is considered flexible when it moves independently of the sperm head (sperm tail flexibility is considered a marker of sperm viability [28, 29]). If tail remains rigid upon touching and sperm head and tail move together as a unit, then spermatozoon is considered non-viable for ICSI.
- Repeat these steps until sufficient number of viable sperm is selected for ICSI.

26.2.6.3 Motility Stimulant Sperm Challenge (MSC) [30–32]

Note: Example given using a 5-mM Pentoxifylline (PF) solution [30] (Appendix).

- Load a 4-μL aliquot of fresh or cryopreserved PESA/MESA/TESA/TESE sperm suspension into the motility stimulant solution microdroplet and incubate for 20 min (Fig. 26.3).
- Examine the specimen microscopically to search for moving sperm. In cases of a positive MSC, a slight noticeable tail twitching is often seen (in rare occasions vigorous twisting may be observed).

- Pick up motile sperm using the microinjection pipette and transfer to a fresh microdroplet of sperm medium. Repeat this step 3–4 times to wash out any residual PF solution (PF was shown to be embryotoxic in animal studies [33], but is apparently safe if used only on sperm [32]).
- Keep selected spermatozoa in culture or place them into a PVP droplet for sperm selection and immobilization for ICSI.
- Repeat these steps until sufficient number of viable sperm is selected for ICSI.

26.3 Expert Commentary

PESA and TESA are effective surgical sperm retrieval methods for men with OA regardless of the cause of obstruction. However, strict criteria to diagnose OA are essential for obtaining a high success retrieval rate in the range of 90–100% [34, 35]. Using PESA, our approach is to perform the first aspiration at the epididymis corpus and proceed to the caput if necessary, since aspirates from the cauda are usually rich in poor-quality senescent spermatozoa, debris, and macrophages [15]. Based on our results, the cumulative sperm retrieval rate (SRR) by using PESA and PESA+TESA was 97.3% [36]. SRR did not differ among men with congenital bilateral absence of vas deferens (CBAVD; SRR = 100%) and vasectomy (SRR = 96.6%) and post-infection (SRR = 96.3%) groups (Table 26.1). In the study mentioned above involving 146 men with OA, the success of PESA was higher in the CBAVD group than in the vasectomy and post-infection groups (96.8% vs 69.5% and 76.4%, respectively, $p < 0.001$).

Most cases of PESA failures are not necessarily technical failures because immotile spermatozoa are found. However, epididymis fibrosis due to multiple PESA attempts or post-infection may render PESA ineffective to retrieve sperm. In such cases, PESA can be attempted at the contralateral epididymis or TESA can be applied successfully [35, 36]. We routinely perform sperm retrieval for OA (PESA/MESA/TESA) under local anaesthesia with intravenous sedation, either on the same day of oocyte retrieval or before if an intentional cryopreservation is planned [14, 37]. Patients are discharged 1 h later and can return to normal activities in the same day. Oral analgesics are prescribed, but pain complaint is minimal. The most common complication is fibrosis at the aspiration site. Other potential complications include haematoma, bleeding, and infection but are very rare [8]. Some authors advocate that MESA allows the collection of larger and cleaner quantities of sperm than PESA (reviewed by Miyaoka and Esteves) [35]. In our series of 146 men with OA, cumulative successful retrieval rate after PESA and TESA was 97.3% (Table 26.1), and an adequate number of motile sperm for cryopreservation were obtained in approximately 26.7% (39/146) of the cases. Although the

Table 26.1 Sperm retrieval rates (SRR) of percutaneous retrievals in obstructive azoospermia according to the cause of obstruction

	CBAVD[a] (*N* = 32)	Vasectomy[b] (*N* = 59)	Post-infection[c] (*N* = 55)	*P* value
Mean ± SD male age (years)	34.6 ± 4.6	46.7 ± 6.7	42.9 ± 10.1	<0.001 (Kruskal-Wallis) ([a]vs[b, c]; [b]vs[c])
Mean ± SD duration of infertility (years)	3.2 ± 2.3	6.46 ± 5.6	4.7 ± 2.1	0.003 (Kruskal-Wallis) ([a]vs[b, c])
Mean ± SD male endocrine profile				
FSH (mIU/mL)	2.3 ± 0.5	6.6 ± 2.5	4.5 ± 2.0	<0.001 (Kruskal-Wallis) ([a]vs[b, c]; [b]vs[c])
LH (mUI/mL)	2.9 ± 0.9	4.7 ± 1.1	3.9 ± 1.5	<0.001 (Kruskal-Wallis) ([a]vs[b, c]; [b]vs[c])
Total testosterone (ng/dL)	631.4 ± 170.5	459.9 ± 163.4	533.2 ± 169.2	<0.001 (Kruskal-Wallis) ([a]vs[b, c]; [b]vs[c])
No. of successful sperm retrievals* (%)				
PESA only†	31 (96.8)	41 (69.5)	42 (76.4)	0.001 (Fisher's exact test) ([a]vs[b, c])
Cumulative (PESA + TESA)	32 (100.0)	57 (96.6)	53 (96.3)	0.68 (Fisher's exact test)
No. of complications (%)	1 (3.1)	4 (6.8)	3 (5.5)	0.90 (Fisher's exact test)
No. of cryopreservation of excess sperm retrieved (%)	7 (21.9)	17 (28.8)	15 (27.3)	0.77 (Pearson chi-square test)

*Successful retrievals defined at obtaining motile sperm

†In a logistic regression model adjusting for male age, duration of infertility, and serum levels of FSH, LH, and testosterone, the overall comparison of SRR by PESA among groups continued to demonstrate statistical significant difference ($P = 0.021$), as well as the pairwise comparisons. No covariate adjustment was performed for analyses of cumulative SRR due to the very high success rates for this outcome

cryopreservation rate after PESA is lower than MESA, repeated aspirations can be carried out in men with OA at a lower cost and morbidity rate than MESA. However, MESA has the advantage of allowing the collection of a greater number of sperm that can be intentionally cryopreserved for future use, thus avoiding the need of repeat retrievals [38]. Moreover, MESA should be the preferred approach for men presenting with coagulopathies.

When gradient centrifugation is chosen for PESA sperm processing, we recommend that part of the sample is spared and processed by simple washing [39]. The reason is the unpredictability of gradient centrifugation to recover motile sperm in such cases. If recovery is suboptimal, we rely on the washed sample to select motile sperm for ICSI. Due to the relatively low sperm yields in PESA and TESA, it is important to use low volumes of media during sperm processing and wash the sample only once. Centrifugation force and time should be carefully controlled to avoid jeopardizing the often-compromised sperm motility.

In a study comparing SRR between OA and NOA, we found that the chances of obtaining sperm were markedly reduced among men with NOA compared with OA, even when micro-TESE was used as the method of sperm acquisition (Table 26.2) [40].

Table 26.2 Sperm retrieval rates (SRR) in azoospermic men with testicular failure and obstructive azoospermia

	Testicular failure	Obstructive azoospermia[a]	*p* value
Number of patients	365	146	–
Male age (years)	36.9 ± 7.6	42.6 ± 9.0	<0.001
Male endocrine profile:			
FSH (mIU/mL)	16.7 ± 10.7	4.8 ± 2.6	<0.001
LH (mIU/mL)	7.9 ± 5.5	3.9 ± 1.5	<0.001
Total testosterone (ng/dL)	412.6 ± 168.1	542.1 ± 186.4	<0.001
Combined left and right testicular volume (mL)	28.7 ± 8.1	37.4 ± 3.5	<0.001
Successful sperm retrieval,[b] *n* (%)	151 (41.4)[c]	146 (100.0)[c]	<0.001
Complication, *n* (%)	12 (6.3)	8 (5.5)	0.76
Excess retrieved sperm cryopreservation, *n* (%)	34 (22.5)	39 (26.7)	0.42

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Data are means ± SD unless otherwise indicated
Wilcoxon Rank Sum Test, Pearson chi-square test, and Fisher exact test were used for comparisons
[a]Previously reported (Esteves et al. [36])
[b]Defined at obtaining sperm
[c]$P < 0.001$ when adjusting for male age and serum levels of FSH, LH, and testosterone in a logistic regression model

As for NOA, our preference is to use *micro-TESE* as the sperm retrieval method [16]. Micro-TESE allows the identification of enlarged seminiferous tubules more likely to harbour sperm production. The minimal tissue extraction and preservation of intratesticular blood supply are important features of micro-TESE, thus reducing the risk of testicular devascularization (reviewed by Esteves [41]). Given the fact that micro-TESE is an invasive procedure, we used strict criteria for diagnosing NOA patients [14, 41, 42]. Our method includes semen analysis results, history and physical examinations, endocrine profiles, and genetic testing. Also, we confirm NOA by histological evaluation. The combination of these parameters was shown to be highly accurate to diagnose NOA.

Furthermore, the use of operating microscope during TESE limits the risk of vascular injury and optimizes the chances of finding sperm [10–12]. Although no absolute predictors for sperm retrieval are available in NOA cases, the probability of retrieving sperm varies according to the testicular histopathology results [12, 13, 43]. Proper identification of testicular vessels under the tunica albuginea is made prior to the placement of an incision into the testis. The microsurgical approach allows preservation of intratesticular blood supply, as well as identification of tubules more likely to harbour sperm production [16]. Therefore, efficacy of sperm retrieval is improved while the risks of large tissue removal are minimized. Excision of large biopsy samples in conventional TESE has been shown to impair testosterone production [44]. Tissue removal in micro-TESE is often 50- to 70-fold less than standard TESE [11], and the small amount of tissue extracted facilitates sperm processing. Selection of spermatozoa from a smaller population of contaminating testicular cells allows more ease and greater speed for sperm pick-up and injection process, as well as alleviates contamination and blockage of the injection needle with cells and debris. It is far less technically demanding and labour-intensive to extract spermatozoa from small-volume specimens than large pieces of testicular tissue that must be dissected, red blood cells lysed, and the rare spermatozoa searched for in a tedious fashion under an inverted microscope (Fig. 26.8). In fact, TESE sperm processing may be incredibly labour-intensive and the searching process may miss the rare spermatozoa within a sea of seminiferous tubules and other cells. TESE/micro-TESE can be scheduled either on the day of oocyte collection (or the day before) or as an elective pre-IVF procedure. In the former, processed specimens are incubated in a closed HEPES-buffered culture system (microdrops under mineral oil) at room temperature, inside a laminar flow cabinet or in a clean

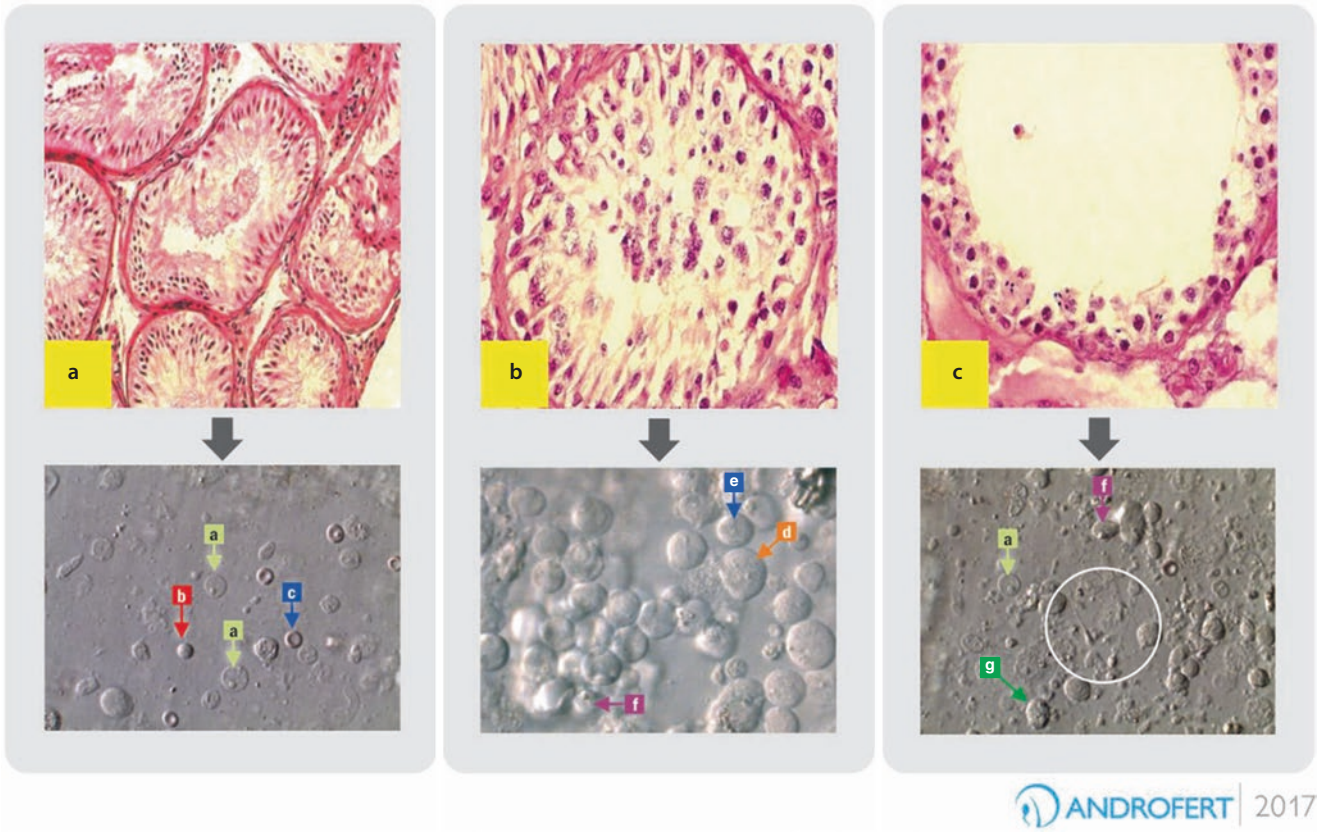

Fig. 26.8 Photomicrographs illustrating the association between testicular histology and cell pattern observed after testicular parenchyma processing. In the upper part, the three main histological patterns found in cases of non-obstructive azoospermia are shown: **a** Sertoli cell only (SCO); **b** maturation arrest (MA); **c** hypospermatogenesis (HYPO). At the bottom is the corresponding cell pattern usually found in each of these conditions as seen under the inverted microscopy. In SCO, only Sertoli cells (a), lymphocytes (b), and erythrocytes (c) are usually observed. In MA, the presence of a large number of germ cells is seen, and cell differentiation usually stops before sperm formation: (d) spermatogonia (e) primary spermatocytes, (f) round spermatids (observe the soft contour and prominent acrosome vesicle in the apical portion). It is important to highlight that isolated foci of active spermatogenesis with differentiation until spermatozoa can be found in SCO and MA. In HYPO, the presence of germ cells is reduced, but all the stages of spermatogenesis are present, including spermatozoa: (a) Sertoli cells, (g) secondary spermatocyte, (f) round spermatid and spermatozoon (highlighted). Magnification: 400×, inverted optical microscope, Nikon Eclipse Diaphot 300 with phase contrast (Hoffman) and haematoxylin/eosin stained for histological slides at 1000× magnification

room for a maximum of 48 h, to avoid bacterial contamination. In the latter, testicular sperm are frozen in special devices (cell sleepers) developed for cryopreservation of low sperm quantity [20]. Culture of specimens at 37 °C inside the incubator should be avoided since contamination with scrotum skin-derived bacteria is often seen [39]. From our data, optimal fertilization by ICSI using surgically retrieved sperm is obtained when the time frame from hCG administration to microinjection does not exceed 44 h [45]. Testicular tissue sperm processing, searching, and selection of viable spermatozoa for ICSI may take several hours in NOA cases. Our laboratory takes approximately 11.6 min to handle a single testicular spermatozoon from processing to microinjection in NOA, but only 5.5 min in OA. In other words, the average time required to perform ICSI in a standard NOA treatment cycle involving 8–12 metaphase II oocytes is approximately 2 h. Therefore, we usually perform micro-TESE the day before oocyte collection when a busy next-day IVF laboratory workload is anticipated. Additionally, we recommend that a minimum of two laboratory technicians work together during the initial processing steps (one mincing the tubules and the other searching for spermatozoa) to speed up the searching process and to allow a faster feedback to the surgeon who may decide to end the procedure if sperm is found or to continue dissecting the seminiferous tubules. Our laboratory performs the processing of testicular specimens by mincing and shredding the whole tissue. If spermatozoa are not found at initial search, a subsequent processing step involves enzymatic digestion using collagenase. The mechanical preparation has the advantage of being fast, requiring about 15–30 min, while enzymatic digestion is more time consuming, requiring at least 2 h [46]. Studies comparing the techniques are scarce, but the existing data were unable to confirm the superiority of one technique over the other for processing fresh and frozen testicular sperm for ICSI [46–49].

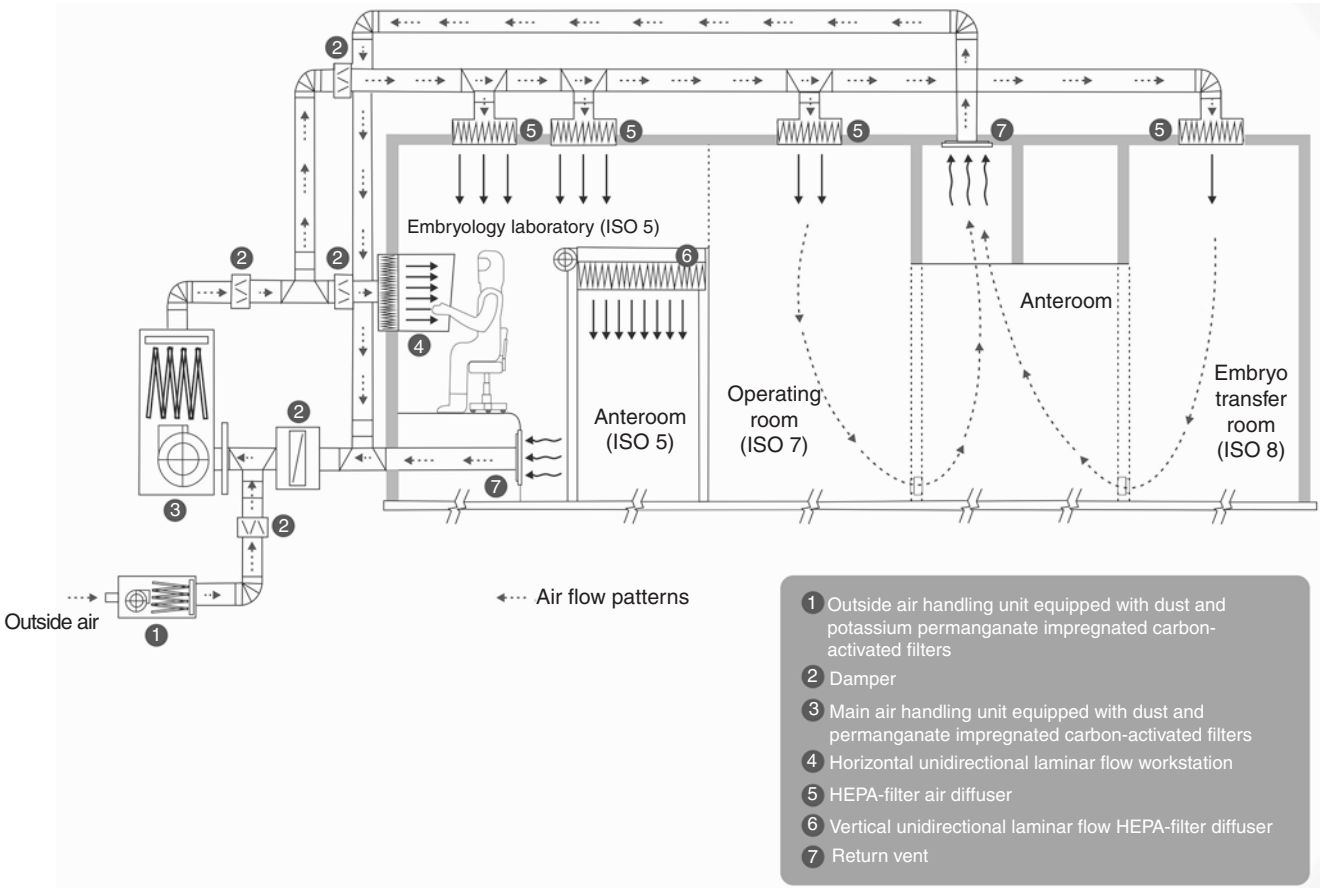

Fig. 26.9 Schematic representation of cleanroom IVF facilities, including airflow patterns and filtration units. The air handling ventilation unit room has a roof-top air-handling unit that draws outside air through coarse and charcoal prefilters before it enters into the main ventilation unit. A free-standing main ventilation unit pulls prefiltered outside air and cleanrooms' return air through coarse filters, past a 16-unit potassium permanganate impregnated pelletized coal-based activated carbon filters, and then through fine dust filters. Lastly, filtered air enters the cleanrooms through high-efficiency particulate air (HEPA) filter diffusers. Floor and ceiling-level vents in the cleanrooms' return air to the main ventilation unit, to be remixed with the existing air. Differential positive pressure is maintained between rooms. The embryology laboratory/anteroom is positive to the operating room, which is positive to both the embryo transfer room and the dressing room/hallways. (Reprinted from Esteves and Bento [50], with permission from Elsevier. (This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License, which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited))

At our centre, sperm retrieval, laboratory processing of PESA/MESA/TESA/TESE specimens, and micromanipulation are carried out in clean room laboratories with particulate and volatile organic compounds (VOC) removal (Fig. 26.9). A detailed description of our clean room facilities can be found elsewhere [51–53]. Evaluating results over a 9-year period, we demonstrated the benefit of operating under these optimum environmental conditions, which resulted in not only an increase in live births but also reduction in miscarriage rates.

In NOA, SRR varies according to testicular histopathology. Patients with maturation arrest (MA) had lower SRR compared with those with Sertoli cell only (SCO). Both categories had lower SRR compared with hypospermatogenesis (Table 26.3) [54]. On the contrary, efficiency of TESA for retrieving spermatozoa for ICSI in NOA is only 10–30% [55], except in the favourable cases of men with testicular histopathology showing hypospermatogenesis, to whom retrieval rates are approximately 80–100% [13]. Nonetheless, if a previous TESA attempt was successful for retrieving spermatozoa in a man with NOA, its positive predictive value for a successful second attempt is 70% [56].

Sperm cryopreservation is routinely used in association with sperm retrieval procedures at our centre. Lately, we prefer to retrieve and intentionally cryopreserve sperm for future use. This strategy offers the advantage of avoiding ovarian stimulation when no sperm is obtained from testicular specimens. If sperm is found and frozen, thawing can be done at any time, thus obviating the need to organize two operations (oocyte and sperm retrieval) on the same day. Also, cryopreservation is useful for surplus specimens that would be discharged after ICSI, in particular, if ICSI fails. Future ICSI attempts may be carried out without repeated surgical retrievals.

Table 26.3 Sperm retrieval rates (SRR), live birth, and obstetrical outcome of resulted offspring according to testicular histology results in patients with non-obstructive azoospermia

	Hypospermatogenesis	Maturation arrest	Sertoli cell-only	*p* value
Number of patients	84	67	205	–
Male age (years)	38.1 ± 9.7	36.4 ± 3.8	36.4 ± 7.2	0.50
SRR[a], *n* (%)	84 (100.0)	27 (40.3)	40 (19.5)	<0.001[b]
Live birth, *n* (%)	20 (23.8)	6 (22.2)	4 (10.0)	0.004[c]
Neonates born, *n*	31	11	6	–
Gestational age (weeks)	36.3 ± 3.2	35.9 ± 1.9	35.5 ± 3.9	0.18
Birth weight (g)	2987 ± 477	2629 ± 870	2583 ± 775	0.12

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Data are means ± SD unless otherwise indicated
Kruskal-Wallis, Pearson chi-square test, and Fisher exact test were used for comparisons
[a]Defined at obtaining sperm
[b]$P < 0.001$ when adjusting for male age and serum levels of FSH, LH, and testosterone in a logistic regression model
[c]$p = 0.01$ when adjusting for co-variates including female and male age, male endocrine profile, duration of infertility, associated female infertility factor, and number of transferred embryos

In OA, motile sperm are usually available after thawing in such cases, and ICSI outcomes using motile fresh or frozen epididymal sperm seem not to differ [57]. On the contrary, motile post-thaw testicular sperm is not always available, and ICSI fertilization rates seem to be slightly lower when immotile testicular sperm are used than both motile frozen-thawed and fresh counterparts [58]. Notwithstanding, methods for selecting immotile viable sperm for ICSI are available. Still, HOST may not discriminate viable and non-viable frozen-thawed spermatozoa [27], and response to motility stimulants is unpredictable. Furthermore, STFT has not been validated in large series [28, 29]. The application of a single laser shot to the far end of the sperm tail has been shown to cause a curling of the tail only in viable sperm, similar to the reaction observed with the HOST, but this method has not been validated in cryothawed specimens [59].

Our first choice as regards handling immotile sperm for ICSI is to perform short-term incubation with culture media. Sperm culture media have the components to support normal metabolism of immotile mature retrieved spermatozoa that may become motile by incubation [60]. If no motile sperm are seen after 2–3 h of incubation, we use pentoxifylline as a motility stimulant. Lastly, we apply the STFT for discriminating viable and non-viable immotile sperm for ICSI after failed pentoxifylline stimulation.

We recently introduced a new method for freezing low sperm quantities, the cell sleeper method, as already described [18, 19]. Ready-to-use TEST-yolk buffer-free cryoprotectants is our preference when freezing sperm by the "cell sleeper" method, as it allows a clean and transparent microdroplet that aids in the process of finding spermatozoa after thawing.

As for the ICSI outcomes in azoospermic men with obstructive and non-obstructive azoospermia, our group has contributed a large amount of data to the literature [36, 40, 43, 54, 61–63]. In one study, the normal two-pronuclear zygote (2PN) and high-quality embryo rates were lower in patients with NOA (47.0% and 43.3%, respectively) than donor sperm (61.0% and 66.5%, $p < 0.001$) and OA (64.0% and 60.9%, $p < 0.001$) groups, respectively (Table 26.4). The mean number of transferred embryos was similar among the groups. Live birth rates were lower among men with NOA (19.9%) than in donor sperm (37.5%, $p = 0.003$) and OA (34.2%, $p < 0.001$) groups, whereas miscarriage rates did not differ among the groups.

Our data indicate that testicular spermatozoa of men with severely impaired spermatogenesis, like those with NOA, have decreased fertility potential. Such sperm may have a higher tendency to carry deficiencies related to the centrioles and genetic material, which ultimately affect the capability of the male gamete to activate the egg and trigger the formation and development of a healthy zygote and a viable embryo [63].

Lastly, we assessed obstetric and short-term neonatal outcomes of babies born after ICSI from azoospermic fathers and compared the results with our population of babies born from non-azoospermic men. In this study, 326 live births resulted from 1041 fresh embryo transfers (live birth rate of 31.3%). A total of 427 babies were delivered and assessed (Table 26.5). Overall, differences were not observed among groups for gestational age and birth weight

Table 26.4 ICSI outcome in azoospermic men with testicular failure, stratified by successful and failed sperm retrieval (donor sperm), and obstructive azoospermia

	Testicular failure	Donor sperm	Obstructive azoospermia[a]	*p* value
Number of cycles	151	40	146	
Female:				
Age	32.7 ± 5.4	31.4 ± 3.5	32.5 ± 5.8	0.32
Basal serum FSH (IU/L)	5.6 ± 2.7	4.6 ± 1.8	5.8 ± 3.0	0.07
Infertility duration (years)	4.3 ± 3.1	3.6 ± 2.2	5.1 ± 4.1	0.08
Number of oocytes:				
Retrieved	13.7 ± 4.4	12.7 ± 6.7	12.3 ± 7.9	0.16
Metaphase II	10.0 ± 5.7	11.5 ± 4.2	9.9 ± 6.1	0.07
Two pronuclei fertilization (%)	47.0 ± 30.0	61.0 ± 17.0	64.0 ± 22.0	<0.001[b]
Embryos:				
High quality (%)	43.3 ± 35.0	66.5 ± 24.3	60.9 ± 39.2	<0.001[c]
Transferred (*n*)	2.7 ± 1.4	2.6 ± 1.5	2.8 ± 1.3	0.51
Clinical pregnancy, *n* (%)	42 (27.8)	20 (50.0)	67 (46.9)	0.002[d*]
Miscarriage, *n* (%)	12 (28.6)	5 (25.0)	16 (23.9)	0.88
Live birth, *n* (%)	30 (19.9)	15 (37.5)	50 (34.2)	0.004[e*]

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Data are means ± SD unless otherwise indicated; Kruskal-Wallis, Pearson chi-square test, and Fisher's exact test were used for comparisons; Donor sperm vs. OA not significant in all pairwise comparisons

*$p < 0.01$ when adjusting for co-variates including female and male age, male endocrine profile, duration of infertility, associated female infertility factor, and number of transferred embryos

[a]Previously reported (Esteves et al. [36])

[b]Testicular failure vs. donor sperm ($p = 0.003$; Wilcoxon Rank Sum Test) and OA ($p < 0.001$; Wilcoxon Rank Sum Test)

[c]Testicular failure vs. donor sperm and OA ($p < 0.001$; Wilcoxon Rank Sum Test)

[d]Testicular failure vs. donor sperm ($p = 0.008$; Fisher's exact test) and OA ($p = 0.002$; Pearson chi-square test)

[e]Testicular failure vs. donor ($p = 0.001$; Pearson chi-square test) and OA ($p = 0.003$; Fisher's exact test)

after controlling for parity. Likewise, the rates of preterm birth, low birth weight, and very low birth weight did not differ among the groups. The preterm birth rates were greatest for singletons in OA (17.9%) and for twins in both OA (47.1%) and NOA (44.5%) compared with the ejaculated group (9.7% and 27%, respectively), albeit differences were not significant. We noted a tendency ($p = 0.06$) towards lower gestational age for twins in the OA group (35.6 ± 2.8) than in NOA (36.2 ± 2.4) and ejaculated groups (37.0 ± 2.3), but the numbers were relatively small to reach statistical significance. The overall perinatal death and malformation rates were 2.8% and 1.6%, respectively, and again the results did not differ among groups. The frequency of babies of the male gender was higher in the OA group (56.4%) compared with the NOA (41.4%) and ejaculated (39.7%) groups ($p = 0.02$).

In another study, we evaluated the neonatal profile of babies delivered after ICSI using testicular sperm from men with NOA and OA. Gestational age, birth weight, and sex ratio of these children were comparable to those reported in a control group that used donor sperm for ICSI. The overall adverse neonatal outcome in the studied population was 4.1% (Table 26.6). Our data suggest that gestational age, birth weight, perinatal death, and malformation rates do not seem to be affected by azoospermia. Although our results on pregnancy and postnatal ICSI outcomes using non-ejaculated sperm are reassuring, the limited population analysed calls for continuous monitoring.

Table 26.5 Neonatal outcomes of children born following sperm injection in azoospermic and non-azoospermic infertile males

	Obstructive azoospermia	Non-obstructive azoospermia	Ejaculated sperm	*P* value
No. of live birth singletons	67	32	145	
Mean ± SD gestational weeks at birth	37.5 ± 2.2	37.8 ± 2.1	38.0 ± 2.1	0.11
No. of preterm birth (%)	12 (17.9)	3 (9.4)	14 (9.7)	0.10
Mean ± SD birth weight (g)	2963 ± 480	2957 ± 667	3092 ± 579	0.24
No. of low birth weight (%)	7 (10.5)	3 (9.4)	10 (6.9)	0.37
No. of very low birth weight (%)	2 (2.9)	2 (6.2)	4 (2.8)	0.38
No. of live birth twins	17	9	37	
Mean ± SD gestational weeks at birth	35.6 ± 2.8	36.2 ± 2.4	37.0 ± 2.3	0.06
No. of preterm birth (%)	8 (47.1)	4 (44.5)	10 (27.0)	0.15
Mean ± SD birth weight (g)	2261 ± 594	2357 ± 403	2461 ± 67	0.30
No. of low birth weight (%)	11 (64.7)	6 (66.6)	18 (48.7)	0.28
No. of very low birth weight (%)	2 (11.7)	1 (11.1)	4 (10.8)	0.92
No. of live birth triplets	5	4	9	
Mean ± SD gestational weeks at birth	32.6 ± 3.1	32.3 ± 5.9	32.6 ± 4.5	0.93
No. of preterm birth (%)	4 (80.0)	2 (50.0)	2 (22.2)	0.37
Mean ± SD birth weight (g)	1660 ± 624	1311 ± 471	1600 ± 642	0.35
No. of low birth weight (%)	4 (80.0)	3 (75.0)	8 (77.8)	0.87
No. of very low birth weight (%)	2 (40.0)	3 (75.0)	3 (33.3)	0.32
Total no. of children born	117	63	247	
No. of perinatal deaths*	3 (2.5)	4 (6.3)	5 (2.0)	0.10
Gender				
No. of boys (%)	66 (56.4)[a]	26 (41.4)[b]	98 (39.7)[c]	0.02 [a]vs[b, c]
No. of girls (%)	43 (36.8)	35 (55.5)	122 (49.4)	0.02 [a]vs[b, c]
No. of unknown (%)	8 (6.8)	2 (3.1)	27 (10.9)	0.06
No. of birth defects (%)	2 (1.7)	2 (3.2)	3 (1.2)	0.26

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Birth defects were defined as structural, functional, or developmental abnormalities presented at birth or later, due to genetic or non-genetic factors acting before birth

*Perinatal death included stillbirths (birth of fetuses with no sign of life that occur after 20 weeks of gestation) and neonatal deaths (deaths within the first 28 days); one stillbirth occurred in each group

Table 26.6 Outcome of neonates born after ICSI in azoospermic men with testicular failure, stratified by successful and failed sperm retrieval (donor sperm), and obstructive azoospermia

	Testicular failure	Donor sperm	Obstructive azoospermia[a]	*p* value
Deliveries (%):				0.49[b]
Singletons, *n* (%)	18 (58.1)	10 (62.5)	39 (76.5)	
Twins, *n* (%)	9 (29.0)	4 (25.0)	10 (19.6)	
Triplets, *n* (%)	4 (12.9)	2 (12.5)	2 (3.9)	
Neonates born, *n*	48	24	65	–
Gestational age (weeks)	36.1 ± 3.6	36.7 ± 2.6	36.3 ± 3.0	0.94
Birth weight (g)	2962 ± 390	2954 ± 498	2978 ± 447	0.99
Proportion male	0.56 ± 0.45	0.58 ± 0.44	0.55 ± 0.457	0.97
Perinatal death,[c] *n* (%)	1 (2.1)	0	1 (1.5)	0.46
Malformation, *n* (%)	1 (2.1)	0	1 (1.5)	0.46

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Data are means ± SD unless otherwise indicated; Kruskal-Wallis and Fisher's exact test were used for comparisons
[a]Previously reported (Esteves et al. [36])
[b]In a Poisson regression model for the number of babies born and adjusting for male and female age, duration of infertility, and serum levels of FSH, LH, and testosterone in addition to female factor infertility and number of transferred embryos, the overall comparison among the groups continued to demonstrate no statistically significant difference
[c]Includes stillbirth and neonatal deaths with frequency calculated as number of perinatal deaths/number of neonates born

Glossary

Azoospermia Absence of spermatozoa in the microscopic examination of the seminal fluid after centrifugation on at least two separate occasions.

Cryopreservation The freezing process for storage of gametes or gonadal tissue at ultra-low temperature.

ICSI *I*ntracytoplasmic *S*perm *I*njection: a procedure in which a single spermatozoon is injected into the oocyte cytoplasm.

MESA *M*icrosurgical *E*pididymal *S*perm *A*spiration: a microsurgical procedure used to aspirate spermatozoa directly from the epididymal tubules for use in an ICSI procedure.

Micro-TESE *Micro*dissection *Te*sticular *S*perm *E*xtraction: a microsurgical procedure used to dissect the seminiferous tubules within the testis in an attempt to identify areas of sperm production and extract spermatozoa for use in an ICSI procedure.

PESA *P*ercutaneous *E*pididymal *S*perm *A*spiration: a procedure in which a needle is inserted into the epididymis to retrieve spermatozoa for use in an ICSI procedure.

Sperm Processing Laboratory techniques used to remove contaminants (cellular debris, microorganisms, red blood cells, etc.) and to select the best-quality spermatozoa to be used in conjunction to assisted reproduction technology.

TESA *Te*sticular *S*perm *A*spiration: a procedure in which a needle is inserted into the testis in order to retrieve spermatozoa for use in an ICSI procedure.

TESE *Te*sticular *S*perm *E*xtraction: operative removal of testicular tissue in an attempt to collect sperm for use in an ICSI procedure.

Appendix

- *Erythrocyte Lysing Buffer Solution (ELBS):* 155 mM NH_4Cl + 10 mM $KHCO_3$ + 2 mM EDTA dissolved in sterile water. Adjust the pH to 7.2, if necessary. Upon finishing the first dilution and centrifugation step, resuspend the pellet with 2.0 mL of ELBS and keep the mixture at room temperature for 10 min. Then, centrifuge the sample at ×300 g for 5 min, discharge the supernatant, and resuspend the pellet in 0.2 mL of fresh HEPES-buffered protein-supplemented sperm medium [46].
- *Enzymatic Digestion:* Prepare 1.0 mL of HEPES-buffered Human Tubal Fluid (Modified HTF culture medium, cat.# 90126, Irvine Scientific, USA) supplemented with 5% of Human Serum Albumin (HSA, cat.# 9988, Irvine Scientific, USA) and 2.6 mg of collagenase type IV (Sigma C5138; activity: 378 Units/mg solid) [64, 65]. Upon finishing the first dilution and centrifugation step, resuspend the pellet with 1.0 mL of the enzymatic digestion solution and incubate at 37 °C for 1 h. Homogenize the suspension every 10–15 min during the incubation period to obtain better enzymatic digestion of the tissue. The digested tissue solution is centrifuged for 5 min at 300 g, and the pellet is resuspended in 100–200 μL of Modified HTF culture medium supplemented with 5% of HSA.
- *Hyposmotic Solution (HOS):* Prepare a 150-mOsm/kg HOS solution by dissolving 7.35-mg sodium citrate and 13.51-mg fructose in 1-mL sterile reagent water [27].

Alternatively, a 139-mOsm/kg HOS solution can be prepared by mixing 1-mL sperm medium to 1-mL sterile reagent water [25, 26].

- *Pentoxifylline Solution (PF)*: Prepare a 5-mM solution of PF by dissolving 1.391-mg pentoxifylline (Sigma cat.# P-1784) in 1 mL of HEPES-buffered culture medium [30].

Review Questions

1. What is the difference between PESA, MESA, TESA, TESE, and micro-TESE and when each method is indicated?
2. Which materials, equipment, reagents, and IVF laboratory setup are needed to perform PESA, MESA, TESA, TESE, and micro-TESE?
3. Which additional sperm processing techniques can be used during sperm search to improve the chances of finding sperm in specimens from non-obstructive azoospermic patients?
4. Which are the additional methods for selecting viable sperm for ICSI when only immotile spermatozoa are obtained after PESA/MESA/TESA/TESE/micro-TESE?
5. What are the advantages of using micro-TESE as the method of choice for harvesting sperm in men with non-obstructive azoospermia in preference over TESE or TESA?
6. What is the association between testicular histopathology results and wet tissue examination in retrieved testicular specimens?
7. Is sperm fertilizing potential similar in obstructive and non-obstructive azoospermia?
8. How to optimize sperm cryopreservation in cases in which only a few spermatozoa are recovered?

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Processing of Sperm Samples in HIV-Positive Patients

Rocio Rivera-Egea, Thamara Viloria Samochin, and Marcos Meseguer Escrivá

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27.1 Introduction

Human immunodeficiency virus (HIV) is a retrovirus which is transmitted from one individual to another through blood, semen, vaginal fluid, pre-ejaculate (via unprotected intercourse, including oral or anal sex), and from mother to child (vertical transmission) during pregnancy and delivery, or via breast milk. Approximately 37 million people around the world are infected with HIV type 1 virus [1], and >80% of HIV-infected individuals are of childbearing age [2].

Over time, infection with this virus can cause acquired immunodeficiency syndrome (AIDS). AIDS is a condition in humans in which progressive failure of the immune system allows life-threatening opportunistic infections and cancers to thrive. This disease was defined in 1982 by the Centers for Disease Control and Prevention (CDC) in the USA. The current classification system is based on clinical, immunological, and diagnostic HIV features [3].

Without treatment, the survival time after infection with HIV is estimated to be 9–11 years; however, the emergence of highly active antiretroviral therapy (HAART) has prolonged the survival of HIV patients and has given rise to a new demand from SDC couples aiming at parenthood. Unfortunately, because of the risk of transmission of viral particles through semen, sexual intercourse in these requires use of protection, which eliminates the possibility of achieving pregnancy.

Thus, in the past, the only completely safe options available to fulfill these couples' desire for offspring were adoption or sperm donation. Nevertheless, many couples desire genetically related offspring.

When the male partner is HIV positive, it is essential to treat the infected semen with effective laboratory techniques that not only isolate the best motile spermatozoa but also remove HIV-infected cells from the semen. These males need special assisted reproduction technology (ART) protocols in order to father children without risking transmission of disease to their partners and offspring [4]. These protocols mainly involve treatment of the sperm sample and confirmation of viral absence before any use of the sample.

For HIV, there is a low transmissibility risk, established as 1–3 infections per thousand occurrences of sexual intercourse (or semen exposure) [4–6]. At this time, when the man is HIV positive and the woman is HIV negative, the options available for serodiscordant couples to have children involve a sperm-washing protocol, confirmation of absence of the virus in the washed sperm, and use of appropriate ART, such as intrauterine insemination (IUI) or in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI) [6–8]. The employment of ICSI in these couples is an applicable alternative to diminish this risk when they undergo ART, because a single spermatozoon is injected directly into an oocyte, during which the pellucid zone of the oocyte is penetrated artificially.

Additionally, to prevent any accidental infection of the partner, offspring, and laboratory staff during ART, we need to employ the safest methods available. To this end, several strategies for treatment of SDC males have been developed in recent years to reduce the transmission risk as much as possible while maintaining reasonable and cost-effective pregnancy rates.

Our aim in this chapter is to present the reader with the available information regarding the safety, efficacy, and effectiveness of employing ART for processing of and insemination with sperm cells in SDC couples in which the male is infected with HIV.

27.2 Sperm Washing, Detection of HIV-1 RNA, and Proviral DNA Techniques

HIV belongs to the retrovirus family; they have the faculty to synthesize reverse transcriptase, convert the ribonucleic acid (RNA) form into deoxyribonucleic acid (DNA), and insert their genome, in this manner, into a host. This issue is very relevant to the detection methods employed [9], since we need to demonstrate the absence of HIV in a sperm sample by analyzing both DNA and messenger RNA (mRNA).

Sperm washing was first described in 1992 by Semprini et al. [10] for the purpose of separating motile sperm from the remaining seminal components, using astringent methods to obtain spermatozoa free of HIV DNA and RNA particles, thereby achieving elimination or minimization of the risk of transmission to the mother and baby when sperm cells are employed in ART. Afterward, different techniques had to be used to determine the absence of the virus in samples (Table 27.1) prior to their use.

Several years ago, when washed sperm samples were employed for IUI treatment, immunofluorescence methods to detect antigen p24 were used in the initial detection of HIV-1; at the present time, the routine detection methods for HIV-1 in sperm are based on amplification of well-defined sequences of the viral nucleic acids [10, 12].

These methods have been developed over time. The initially available methods had significant limitations, because their lower detection limit was 10,000 copies per milliliter. The second-generation techniques had a lower detection limit of 200–400 copies [3]. Another method (third-generation) evolved that included a sequence as a control system to demonstrate the efficiency of amplification for each tested sample, with sensitivity low as 50 viral copies [11], but it could not detect viral presence below this threshold.

The most commonly used method of detection is standard polymerase chain reaction (PCR), which consists of enzymatic amplification of specific sequences of the viral genetic material, but this technique also has a lower limit in the number of copies that can be detected (around 200 copies/mL of RNA), making it impossible to be 100% sure that the sample is free of the virus.

Table 27.1 Separation and human immunodeficiency virus (*HIV*) detection techniques used in sperm samples from HIV-positive patients

Study	Sperm washing	Swim-up	HIV semen detection method
Semprini et al. (1992) [10]	Double-density gradient (40–80%)	Yes	Indirect immunofluorescence
Lasheeb et al. (1997) [11]	Double-density gradient (40–80%)	Yes	NASBA nested PCR
Marina et al. (1998) [12]	Triple-density gradient (50–70–90%)	Yes	Amplicor PCR
Chrystie et al. (1998) [13]	Double-density gradient (50–90%)	Yes	NASBA
Hanabusa et al. (2000) [14]	Quadruple-density gradient (56–64–72–80%) in a double tube	Yes	Nested PCR
Sauer and Chang (2002) [15]	Double-density gradient (47–90%)	Yes	None
Politch et al. (2004) [16]	Double-density gradient (47–90%) in a double tube	No	RT-PCR
Garrido et al. (2005) [17]	Triple-density gradient (45–70–90%)	Yes	Nested PCR
Mencaglia et al. (2005) [5]	Triple-density gradient (45–70–90%)	Yes	None
Bujan et al. (2007) [18]	Triple-density gradient (50–70–90%)	Yes	HIV RNA PCR
Kato et al. (2006) [19]	Double-density gradient	Yes	Nested RT-PCR
Savasi et al. (2007) [7]	Double-density gradient (40–80%)	Yes	Real-time PCR
Molina et al. (2014) [20]	Double-density gradient (40–80%)	No	Real-time PCR
Zamora et al. (2016) [21]	Triple-density gradient (45–70–90%)	Yes	Real-time PCR

NASBA nucleic acid sequence–based amplification, *PCR* polymerase chain action, *RNA* ribonucleic acid, *RT* reverse transcription

Trying to improve the techniques available, our group developed a protocol to reduce the detection limit to a single RNA or DNA viral copy, using nested PCR (nPCR) or reverse transcription and nPCR for RNA [9]. With application of this method, it has been shown that many samples that were considered negative with other methods are in fact positive. Despite this, no reports of infection after use of different methods have been published.

27.3 Semen Analysis

All samples must be processed in a dedicated laboratory; it is necessary to employ highly trained and experienced staff, and to make use of an exclusive and isolated laboratory area, set apart from work with noninfectious samples, with use of a class II/B3 biosafety cabinet with a vertical laminar flow hood, a centrifuge with safety lids, an exclusive incubator, and a nitrogen tank used solely for storage of these potentially infectious samples.

Samples are collected by masturbation into a sterile polypropylene container after 3–5 days of ejaculatory abstinence. After liquefaction and homogenization, semen parameters are analyzed according to the World Health Organization [6, 22] criteria. The total sperm count and motility before and after the wash are recorded. Sperm morphology is not analyzed for safety reasons: it is not recommended to work with cutting elements when treating HCV-positive/HIV-positive samples, and, as these samples are always used for ICSI treatment, fresh sperm morphology is not needed.

27.4 Sperm-Washing Procedure

The sperm-washing procedure has emerged from a modification of classical sperm preparation methods. Although this procedure can differ in different clinics, the elimination of HIV from raw sperm commonly consists of double and consecutive processes to wash the semen sample by means of modified classical density gradient and swim-up techniques [10], both of which have a single aim: to isolate only motile sperm.

As mentioned earlier, these techniques are also used generally in sperm preparation for human reproduction to select the most suitable motile sperm for fertilization [23], but in this case we use them consecutively and with larger volumes of culture media to perform the procedure more strictly to remove the viral particles completely.

Most authors have applied the original protocol defined by Semprini et al. in 1992, but with small variations in the gradient concentrations [9, 12], double gradients [16], or omission of the swim-up technique [24].

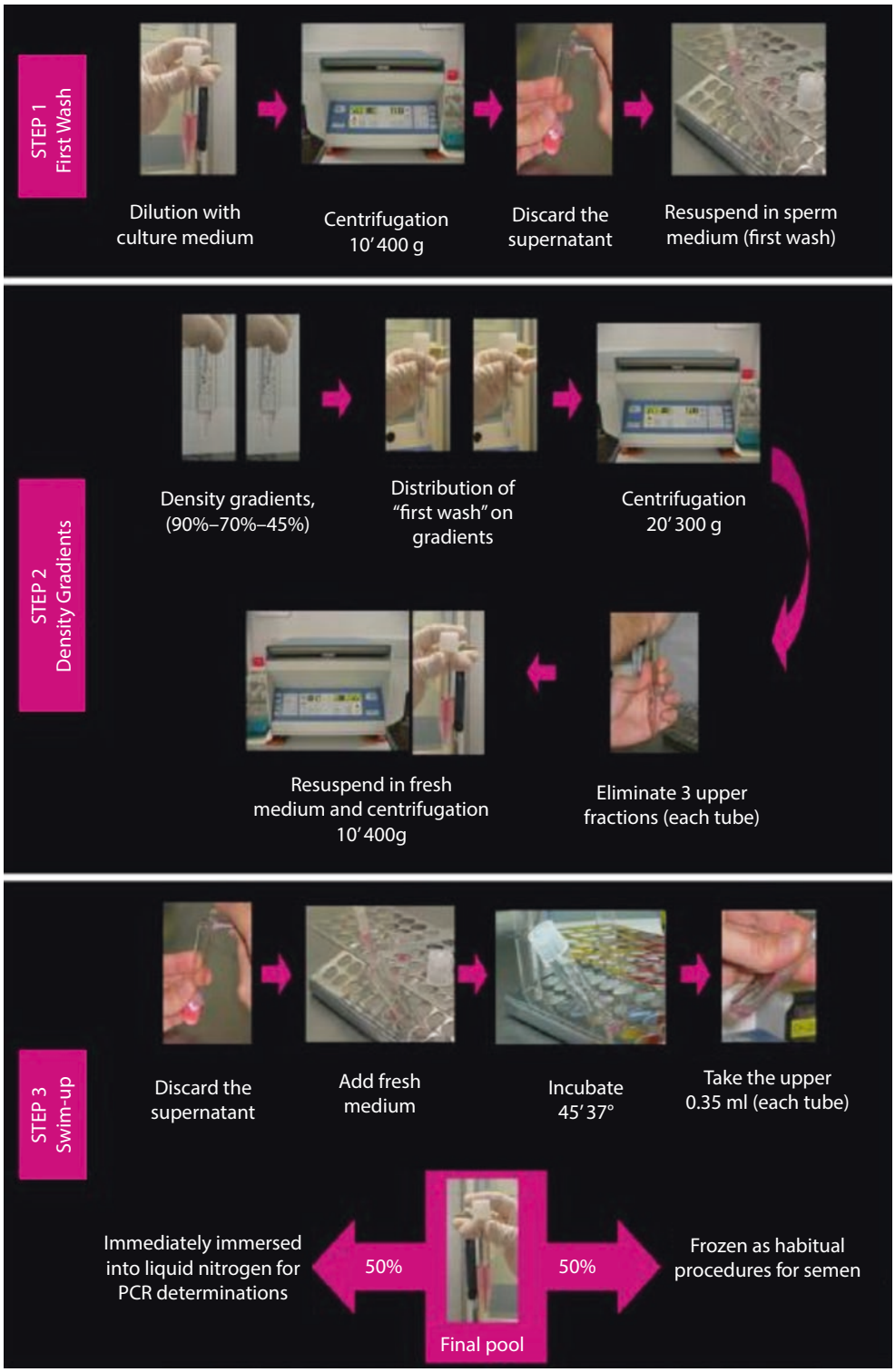

Fig. 27.1 The sperm wash procedure consists of three steps

The sperm-washing process consists of three steps (Fig. 27.1) [9].

Step 1. First wash: seminal plasma removal

- An initial dilution or first wash is performed in about a 1:1 proportion (vol:vol) with culture medium after liquefaction of the ejaculate.
- The sperm is then pelleted at 400 × *g* for 10 min, and the supernatant is discarded.
- The pellets are then resuspended in fresh culture medium (the "first wash").

Step 2. Density gradients: establishment of liquid layers of different densities

- Different conical tubes of triple-density gradients (Percoll®, PureSperm®, SpermFilter®, Ficoll®) with 1–1.2 mL in each layer (90%, 70%, and 45%) are prepared in order to separate different sperm cell fractions, and approximately 1 mL of the first wash is layered into each tube.
- Centrifugation is performed for 20 min at 300 × *g*; as a result, each component of the sperm begins to cross through the different layers until it reaches the layer where the solution density is equal to its own density.
- The upper fractions of the tubes are then eliminated.
- Each pellet is obtained and washed with 5 mL of fresh culture medium and then repelleted again at 400 × *g* for 10 min. The supernatants are discarded.

Step 3. Swim-up: selection of the best motile spermatozoa

- After the supernatant is discarded, a swim-up of 1 mL of fresh medium is done. After 45 min at a 45° angle in the incubator at 37 °C in a 5% CO_2 atmosphere, the upper 0.5 mL in each tube is extracted carefully and pooled in a new tube, meaning that the resulting washed sperm suspension is about 1 mL.
- After evaluation of the total count and motility, one half of the "pool" is immediately immersed in liquid nitrogen for PCR determination, and the other half is frozen with cryoprotectant medium, following the manufacturer's instructions, and stored until use in ART after a negative viral presence test [9].

Obviously, the process of strict sperm washing to eliminate as much of the virus as possible adversely affects the amount of available sperm for IUI or IVF, with recovery of only about 5% of the total number of spermatozoa available in the raw sample [3, 17]. However, our group has modified the usual sperm-washing protocols to be less strict in very poor samples and even in testicular biopsy samples [25, 26].

27.5 PCR Techniques for HIV Applied to Semen

Nucleic acids were extracted from washed spermatozoa using a NucliSens (Organon-Teknika, Barcelona, Spain), following the instructions of the manufacturer. Two extractions were run in parallel, one using a spermatozoa sample and the other using a spermatozoa sample with addition of HIV RNA obtained from HIV-infected plasma to detect the presence of transcription or amplification inhibitors after the nucleic acid extraction procedure. Both extracted samples were used for two HIV RNA transcriptions to detect genes from the *gag* and *pol* region, followed by nested DNA amplification [27]. The same samples were used to amplify HIV proviral DNA by nested amplification to detect both genes (*gag* and *pol*). The other extraction, run in parallel with added HIV RNA before nucleic acid extraction, was used as a positive control to detect the presence of inhibitors of the transcription or amplification. Besides use of negative controls to detect the presence of amplicons, contamination was carried out.

For HIV RNA transcription, the antisense external primers for annealing were used with nucleotides 1696–1676 and 3286–3265 for the *gag* and *pol* genes, respectively. Standardized conditions for transcription were followed, using 100 μM DTT, 1 mM of each dNTP, 0.2 μM antisense primer, 20 U of RNasin (Promega, Barcelona, Spain), and 5 U of AMV transcriptase (Promega) in a final volume of 20 μL. Nested DNA amplification used the external primers to anneal with nucleotides (from the ARV2/SF2 sequence) 1224–1243 and 1696–1676 and internal primers to anneal with nucleotides 1316–1335 and 1524–1504 for the *gag* region. External primers annealing with nucleotides 2623–2642 and 3286–3265 and internal primers annealing with nucleotides 2716–2741 and 3250–3227 for the *pol* region were used [28].

Standardized PCR conditions were followed, including 6 μL of reverse transcriptase (RT) or previous PCR product; 2.5 and 2 mM $MgCl_2$ for the *gag* and *pol* regions, respectively; 0.2 mM of each dNTP (Amersham Pharmacia, Madrid, Spain); 0.5 μM of each primer; and 2 U of Taq polymerase (Promega) in a 50-μL final volume. β-Actin gene amplification was performed to confirm the presence of DNA in the extraction from the spermatozoa suspension. The results were read after 2% agarose gel electrophoresis after ethidium bromide staining [27]. In all of the samples, a consistent result (either positive or negative) was obtained. The assay failure rate was zero.

The quantitative one-round PCR technique was performed according to the method specified by Roche Diagnostic Systems (Amplicor; Roche, Basel, Switzerland) and as described in the work by Marina et al. [12]. Briefly, RNA extraction was performed according to the Amplicor specimen preparation kit specifications (Roche, Barcelona, Spain).

To check for the HIV RNA load, reverse transcription and amplification using SK145 and SKCC1B were carried out (amplifying a limited region of the HIV-1 *gag* gene, which translates the viral proteins p18, p24, and p55). The PCR-amplified product was detected and quantified through hybridization with the use of a specific biotin-labeling probe. An enzyme-linked immunosorbent assay (ELISA) was used for subsequent detection.

The results obtained by Meseguer et al. [9] from the analysis for detection of the presence of viral molecules after the sperm washes demonstrated that approximately 7% of sperm washes still led to positive results afterward. In those cases, the sample was reanalyzed, and the results for the samples that tested positive by nested PCR were compared with those from the one-round PCR protocols, and the results were negative. The authors programmed another sperm wash after 2–3 weeks and never obtained a positive result after a second wash. The assay never failed to yield a diagnostic result.

This undoubtedly indicates that a negative sample in the protocols employing one-round PCR still had an undetectable viral presence [9]. Nevertheless, different institutions employ different methodologies, with highly variable efficiency and detection limits [29, 30].

Currently, we cannot predict the seminal washing results, but experience from different research groups seems to suggest that the rate of positive washes may be around 1–5% [17].

27.6 Use of Assisted Reproduction Techniques in Serodiscordant Couples

At the beginning of the AIDS epidemic, doctors refused to use ART in this population, because of the high morbidity and risk of infection (via horizontal and vertical transmission, or via transmission to medical personnel). However, the introduction of HAART has achieved a delay in the disease, extending the survival and improving the quality of life of people infected with HIV [23].

With this new therapy and its results, the ambition of patients to have children has increased, but, to avoid HIV transmission, these men cannot have unprotected intercourse with their partners. For that reason, doctors have recommended that patients consider sperm donation, adoption, or abandoning their hopes of fatherhood [31]. However, improvements in our knowledge of the disease and ART now allow these couples to have a family. For that, it is necessary to provide adequate reproductive counseling to achieve pregnancy [8].

To choose which treatment (IUI, IVF, or ICSI) should be used when the man is HIV positive and the woman is HIV negative, several aspects must be considered. IUI has been the alternative method used for a period of time, given the low degrees of difficulty and invasiveness [10, 12]. This method may be simpler and less expensive than others, but it presents some important inconveniencies, such as the need for a sperm wash result on the same day; if a positive result is found, the cycle will be canceled. Another problem is the small number of spermatozoa inseminated and the potential risk of viral transmission due to the exposure to thousands or even millions of "potentially infective" spermatozoa. Also, the whole sperm preparation must be inseminated within the same cycle. For these reasons the use of classical IVF or ICSI is recommended [32–34].

ICSI involves a smaller number of sperm cells than IUI (which requires millions of sperm) [15, 32, 33, 35, 36]. Despite the high cost of the ICSI process, the efficiency of this technique avoids the need for expensive repetitive virological semen testing and numerous cycles of ovarian stimulation. Another advantage over IUI relates to the increase in pregnancy rates per treatment cycle (more or less three times higher with ICSI; nearly 50% per cycle versus 17%), which should decrease the number of attempts needed to establish a successful pregnancy.

With ICSI, we are exposing the woman to a nearly infinitesimal risk: for each oocyte we employ just a single sperm obtained from a sample that has previously tested negative for HIV on PCR.

In another method, the semen samples are frozen, and the nPCR results can be confirmed as many times as needed, since the sample is not necessarily employed on the same day. Also, sperm washing can be performed before the cycle, thus not causing cancellation of any cycle because of a positive result. If the sperm-washing result is negative, it can be employed several times as needed, thus avoiding the need for (and expense of) new washes.

Additionally, in men with a priori lower semen quality that will not be sufficient for optimal IUI, ICSI is the only realistic treatment option because after the extensive procedure involved in sperm washing, and to avoid the possibility of a positive HIV result, the sperm recovery is very low (about 5% of the initial motile sperm).

Objectively, we must keep in mind that both techniques have been employed successfully to date to help serodiscordant people succeed in fulfilling their reproductive wishes safely [5, 10, 12, 37–40]; nevertheless, ICSI is the most preferred and safest method used so far [23], although it presents obvious disadvantages in comparison with IUI, such as the side effects associated with the procedure.

27.7 Sperm-Washing Results: Efficiency, Pregnancy, and Seroconversion Rates

Next, we analyze the results of a program for SDC couples in which the male was HIV positive, in terms of seroconversion (HIV transmission to HIV-uninfected women), vertical transmission (HIV transmission to newborns), and pregnancy rates when washed samples were employed.

In this study, our group [4] assessed couples comprising seronegative women and HIV-positive men (n = 18), HIV-positive men with HCV coinfection (n = 33), and infertile couples undergoing ART where the man was HCV positive (n = 40), providing a total number of 134 sperm samples to be washed. HIV infection was acquired by parenteral drug addiction in 25 of these cases (48.8%), plasma donation in 1 case (0.2%), sexual transmission in 11 cases (21.6%), blood transfusion in 6 cases (11.7%), and unknown in 8 cases (15.5%).

With regard to the female population, only those with demonstrated absence of HIV and HCV antibodies were accepted for inclusion in the study. They were also requested to practice sex with condoms.

Different gynecological findings were observed: 59 of the women were considered normal (64.8%), 15 of them were more than 36 years old (16.4%), 5 of them were low responders (5.5%), and 8 of them had endometriosis (8.6%). Depending on the patient characteristics, we treated these women with ICSI with their own oocytes or with oocytes obtained from healthy young donors. In the HIV-SDC couples, 11 procedures were performed with donated eggs, and seven procedures were performed with donated eggs in HCV-SDC couples.

In this work, we found a global pregnancy rate of approximately 45% per cycle [4], and almost 95% of the couples achieved pregnancy within four consecutive cycles.

Table 27.2 Global cycle results depending on the infection

	HIV infection (total)	HIV infection only	HIV + HCV infection	HCV infection
Follicular aspirations	73	27	46	51
Oocytes obtained	920	352	568	621
Metaphase II	742	293	449	514
Zygotes	511	211	300	305
Frozen embryos	169	78	91	68
Transferred embryos	154	59	95	116
Metaphase II/follicular aspiration	9.9 ± 2.3	10.9 ± 2.0	10.2 ± 2.0	10.5 ± 3.0
Fertilization rate/metaphase II, %	68.9 ± 6.3	72.0 ± 8.1*	66.8 ± 6.0	59.3 ± 5.3*
Embryos/follicular aspiration	4.3 ± 2.0	5.1 ± 1.8	4.0 ± 2.0	3.8 ± 0.7
Cleavage rate (embryos/metaphase II), %	57.3 ± 5.3	62.4 ± 5.6	53.4 ± 8.3	44.9 ± 10.2
Embryo transfers	64	23	41	42
Embryos/transfer	2.4	2.6	2.2	2.8
Positive pregnancy test	29	9	20	12
Negative pregnancy test	29	12	17	29
Biochemical pregnancy	5	1	4	1
Newborns	19	4	15	4
Pregnancy rate/cycle, %	46.0	40.1	48.7	40.1
Frozen embryo transfer	10	6	4	8
Frozen embryo positive pregnancy test	2	0	2	0
Frozen embryo negative pregnancy test	8	6	2	6
Frozen embryo pregnancy rate, %	20	0	50	0

Results are expressed as total number or as mean ± standard error of the mean
HCV hepatitis C virus, *HIV* human immunodeficiency virus
*$p < 0.05$

The results are shown in Table 27.2. Blood analyses after 3, 6, and 9 months confirmed no seroconversion in the women.

Recently, Barnes et al. [41] performed a systematic review and meta-analysis to study the efficacy and safety of ART in SDC couples, and found that sperm washing coupled with IUI, IVF, or ICSI was a safe method, with no cases of seroconversion reported.

Two years later, Zafer et al. [42] performed another systematic review and meta-analysis to evaluate the effect of sperm washing on HIV transmission among HIV-SDC couples. They, too, demonstrated that sperm washing was a safe and effective method to be used in positive HIV patients to achieve pregnancy. In their review they found no seroconversion among HIV-uninfected woman inseminated with washed semen, decreasing the risk of HIV transmission so far estimated, and they found no cases of vertical transmission. Moreover, clinical pregnancy was achieved by more than half of the couples included in the review.

Likewise, Zamora et al. [21] demonstrated that sperm washing using triple gradient sperm selection with extensive centrifugation and swim-up was a highly reliable technique for HIV removal, diminishing the frequency of postwash positive tests and maintaining pregnancy rates.

27.8 Influence of HIV-1 Infection on Semen Quality: Basic Sperm Analysis and the Embryo Point of View

As mentioned earlier, semen quality in seropositive men may be affected by different factors, including the HIV infection itself, the individual's overall health status, HAART therapy, or sperm washing [23]. Several studies [17, 43, 44] have focused on the assessment of seminal characteristics in HIV patients to analyze factors that make sperm washing fail and sperm quality in SDC males. Our group has evaluated male infection parameters, antiretroviral treatments received, and

Table 27.3 Receiver operating characteristic (*ROC*) curve analysis results for prediction of postwash polymerase chain reaction (*PCR*) results regarding infectious particles, according to semen parameters and human immunodeficiency virus (*HIV*) patient features ($n = 73$)

Parameter	Standard deviation of the value of area under the curve	Threshold	Sensitivity, %	Specificity, %	PPV, %	NPV, %
CD4 cell count	0.59 [0.469–0.704]	≤147 cells/μL	37.5	92.5	37.5	92.3
Evolution of HIV	0.53 [0.410–0.653]	≤12	85.7	30.2	12.0	95.0
HIV viral load	0.49 [0.289–0.690]	≤44,000 IU/mL	100	36.4	22.2	100
Volume	0.49 [0.397–0.576]	>3.4 mL	61.5	55.2	13.3	92.8
Concentration	0.65 [0.561–0.731]	≤13.6 millions/mL	38.5	87.9	26.3	92.7
Type A + type B motility[a]	0.55 [0.458–0.636]	>67 %	23.1	93.9	30.0	91.5
TMP	0.62 [0.537–0.711]	≤38.07 millions	69.2	73.9	23.1	95.5
Volume of prepared sample	0.55 [0.463–0.642]	≤1.1 mL	69.2	46.5	12.9	93
Concentration postwash	0.57 [0.479–0.658]	>1 mill/mL	100	20.4	12.6	100
Type A + type B motility[a] postwash	0.59 [0.507–0.685]	≤80 %	69.2	57.5	15.8	94.2
TMP postwash	0.68 [0.600–0.769]	≤2.2 millions	84.6	58.9	19.3	97.1
Recovery rate	0.56 [0.467–0.647]	≤0.1541 %	23.1	95.5	37.5	91.5

NPV negative predictive value, *PPV* positive predictive value, *TMP* total motile progressive sperm
[a]According to World Health Organization reference values [22]

sperm quality to identify positive-wash predictors [17]. This study included 73 healthy seronegative males (as a control population), who were partners of women who exclusively had tubal factor infertility, within the same period of time. The controls and HIV-positive males were matched for age (±2 years) and the number of days (±0.5) of sexual abstinence.

Between August 2001 and November 2003, a total of 125 males provided 136 samples to be washed. Their ages ranged between 21 and 54 years (median 37.1 years).

In the 70 HIV-infected males (45 (64.3%) of whom were also coinfected with HCV), the mode of infection was former addiction to parenteral drugs in 32 cases (45.7%), plasma donation in 2 cases (3.0%), heterosexual transmission in 15 cases (21.4%), and unknown in 14 cases (20.0%). The average duration of HIV disease was 9.6 years (range 1–20 years). According to the CDC classification of the disease, 12 cases (17.1%) were A1, 23 (32.8%) were A2, 5 (7.1%) were A3, 3 (4.3%) were B1, 7 (10%) were B2, 11 (15.7%) were B3, 2 (2.8%) were C2, and 6 (8.5%) were C3.

With regard to antiretroviral treatments, 21 of the patients (30%) were treatment free, while the remainder were receiving different treatment combinations: 2 were on a single therapy (2.8%), 4 on two therapies (5.7%), 36 on three therapies (51.4%), 6 on four therapies (8.5%), and 1 on six therapies (1.4%).

The average blood HIV load was 19,408 IU/mL (ranging from undetectable to 525,000 IU/mL), while 39 patients (55.7%) showed a negative viral blood load. The average peripheral blood CD4 cell count was 497.5 cells/μL, ranging from 26 to 1064 cells/μL.

The results of this work showed that there is no systematic way to predict a positive result after sperm washing, as demonstrated by the receiving operator characteristic (ROC) curve analysis of the different factors (Table 27.3). Moreover, we found no significant decrease in sperm quality in the HIV-positive group in comparison with the healthy controls, and the antiretroviral treatments received did not affect the sperm quality; with regard to this last issue, a similar finding was reported by Lopez-Ruz [45] in a recent prospective study. However, other authors have reported changes in semen analysis parameters (volume, total sperm count, and motility) [11, 43, 44].

Also, semen quality can be evaluated in different ways by studying sperm molecular factors related to male infertility [46, 47] or by studying the embryo quality obtained from these sperm samples in comparison with healthy controls [48]. This way of assessing semen quality has been used by Melo et al. [48] and by us. From our data, we concluded that the fertilization and cleavage rates were comparable between the groups. On days 2 and 3 of embryo development, very similar embryo features were found in the different groups. Additionally, there was no difference in the mean numbers of optimal embryos on day 3; however, when the embryos were cultured for up to 5–6 days, a significantly higher rate of embryo blockage was found in the SDC group than in the control group. However, the mean numbers of optimal blastocysts on day 6 were comparable in both groups. With regard to the numbers of cryopreserved and transferred embryos, and the implantation, pregnancy, multiple pregnancy, and miscarriage rates, no differences were found between the groups.

27.9 Is Sperm Washing Possible in Oligospermic or Azoospermic Samples?

For these sperm-washing protocols to be performed, the ejaculate must contain a minimum of two million motile sperm cells.

27.10 Patients with Severe Oligospermia

At our institution, approximately 2% of HIV- or HCV-seropositive men presenting for fertility treatment have severe oligospermia [4]. In these cases, execution of the exhaustive sperm-washing protocols described previously make it impossible to recover any sperm at the end of the procedure.

In males who present with lower sperm counts, we have considered the possibility of performing modified sperm washing, using serial dilutions of the sperm sample, and then confirming the absence of viral nucleic acids by use of PCR.

The first series was published in 2006; out of seven males with severe oligospermia undergoing modified sperm washes, none of the samples tested positive for HIV, and so they were able to be used in ART treatments [25]. This is, to our knowledge, the only published report of ART in oligospermic males with HIV.

27.11 Sperm-Washing Protocol for Oligozoospermic Males

This protocol is very similar to the main protocol but with small modifications.

Ejaculate obtained after sexual abstinence for 3–5 days should be allowed to liquefy for 10 min at 37 °C and then be diluted 1:1 (vol/vol) with culture medium. The mixture is then centrifuged for 10 min at 400 × *g*, and the supernatant is carefully discarded. This procedure has to be repeated twice, and the resulting cells are resuspended in a final volume of 0.5–1 mL.

One half of each sample must be submerged immediately in liquid nitrogen for PCR determination, and the other half must be frozen, as described elsewhere [9], and stored until it is used in ART once a negative viral presence has been confirmed.

27.12 Patients with Azoospermia

An extreme situation is presented by males with no sperm in their ejaculate. Two case reports are available in the literature, describing an epididymal search for motile sperm in azoospermic males presenting with obstructions in the genital tract. In these papers, only patients with epididymal sperm retrieval were presented [49, 50]. This technique could be inappropriate for some azoospermic males. For example, this method could not be applied if the obstruction is up in the genital tract, or in males presenting with nonobstructive azoospermia with no spermatozoa available in the epididymal reservoir.

Open testicular biopsies augment the chances of finding motile sperm cells in azoospermic patients, although the presence of blood and round cells could increase the risk of a viral presence after washing.

Our group has published three cases of open testicular biopsy in patients with azoospermia, which is also applicable to males presenting with nonobstructive azoospermia [26].

27.13 Sperm-Washing Protocol for Azoospermic Males

Samples were obtained from male patients by testicular sperm extraction (TESE) because of the absence of sperm in two consecutive sperm analyses obtained and analyzed between August 2007 and November 2008. All patients had a normal karyotype and no microdeletions on the Y chromosome.

TESE was performed as previously described [51]. Briefly, open testicular biopsies were carried out with a 2% mepivacaine spermatic cord block. After the scrotal skin and tunica vaginalis were opened, three small incisions were made through the tunica albuginea in different regions of each testicle, and small pieces of extruding testicular tissue were excised. Two fragments (one per testis) were placed in Bouin's solution and were sent for histopathological examination. The remaining fragments were taken to the adjacent laboratory for sperm retrieval. The testicular tissue was placed in 2 mL of culture medium and minced mechanically with sterile slides. The presence of sperm cells was checked under an inverted microscope at ×3400 magnification. If motile sperm were found, the samples were centrifuged for 10 min at 400 × *g*, the supernatants were then carefully discarded, and the samples were immediately frozen. This procedure was repeated twice, and the resulting cells were resuspended in a final volume of 0.5–1 mL.

When this initial microscopic evaluation did not show motile spermatozoa, the sperm suspension was transferred into a Falcon tube and centrifuged at 600 × *g* for 5 min. The pellet was resuspended in 0.5 mL of sperm medium and incubated at 37 °C in a 5% CO_2 atmosphere for 1 h. Then, the samples were checked again for the presence of motile sperm.

Continually, for freezing, an equal volume of cryoprotectant culture medium was added to the sperm pellet and the mixture was then homogenized and kept at room

temperature for 10 min. Sperm samples were frozen in small tablets on a dry ice surface for approximately 1 min and were then transferred to prelabeled cryotubes. One half of each sample was immediately submerged in liquid nitrogen for PCR determinations, and the other half was frozen, as previously described, and stored in separate liquid nitrogen tanks until it was used in ART once a negative viral presence had been confirmed.

For thawing, the pills were removed, transferred into 5-mL Falcon tubes, and placed in an incubator at 37 °C in a 5% CO_2 atmosphere. Then the samples were washed again with fresh medium and centrifuged at 600 × *g* for 5 min. The supernatant was discarded, and the samples were resuspended in variable amounts of medium. Thereafter, motile spermatozoa were checked again for ICSI.

After sperm washing, motile sperm were recovered to be frozen from all testicular biopsies, thus guaranteeing safe use of the spermatozoa found within the samples. Two pregnancies were achieved out of five follicular aspirations, with four fresh and two frozen–thawed embryo transfers, resulting in a healthy newborn and an ongoing pregnancy.

Although they were achieved in only a limited sequence of cases, our results confirm that TESE-ICSI treatments in azoospermic seropositive patients are possible, and that these males should not be rejected for ART if these techniques, together with sperm washing and PCR confirmation of viral absence, are available.

27.14 Conclusion

The laboratory is focused on two aspects: first, we need to eliminate any viral particles, since nested polymerase chain reaction (nPCR) is an extremely sensitive method, and any viral residue will be detected, thus yielding a positive result and forcing us to discard the sample; second, we must optimize the sperm-washing procedure to ensure that we retain as many motile sperm as possible, to permit as many intracytoplasmic sperm injection (ICSI) procedures as possible.

In conclusion, sperm washing seems to reduce the risk of transmission in serodiscordant couples in which the man is infected with human immunodeficiency virus (HIV), and it is an effective, safe, and reliable technique to remove viral particles. Thus, HIV-infected patients now have the possibility to become fathers, avoiding viral transmission to the mother and the future child. Moreover, the sperm parameters are not significantly different from those that the World Health Organization considers normal.

From all of this information, we can summarize that sperm washing, with use of nPCR as a confirmatory molecular biology technique, followed by ICSI, is the most suitable procedure to perform in serodiscordant couples in which the man is HIV infected, in order to create a family in a cost-effective and safe way.

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An Emerging Medical Device: Electrophoretic Sperm Separation

Steven Fleming and Robert John Aitken

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Learning Objectives

- Limitations of existing methods of sperm preparation
- Importance of sperm charge in defining gamete quality
- Understanding the basic design of an electrophoretic sperm isolation device
- Appreciation of the efficiency of this device in achieving the rapid efficient isolation of high-quality spermatozoa from the ejaculate

28

28.1 Overview

28.1.1 Principles of Electrophoresis

Electrophoresis is a term used to define particle dispersion within a liquid medium, the electrolyte, in response to a spatially uniform electric field. This electrokinetic phenomenon occurs as a result of the particles displaying a net positive or negative surface charge against which an external electric field can exert an electrostatic force. In fact, a surface charge may not even be necessary for electrokinesis, as it is theoretically possible that even neutral particles could migrate in response to an electric field by virtue of the molecular structure of water at their interface. This concept relates to the so-called double layer theory, whereby a diffuse layer of ions having the same but opposite charge to the particle surface screens them from the surrounding medium. Consequently, the electric field exerts an electrostatic force on the ions within the diffuse layer in the opposite direction to that exerted upon the particles, resulting in viscous stress, termed the electrophoretic retardation force. This hydrodynamic friction applied to the particles depends also upon the viscosity of the liquid medium in which they are dispersed, ultimately determining their electrophoretic mobility. Hence, it is necessary to carefully consider the molecular weight and charge of the particles relative to the conductivity and viscosity of the electrolyte to achieve the electrophoretic mobility required.

28.1.2 Electrophoretic Properties of Spermatozoa

Normal, mature spermatozoa carry a net negative charge that is imparted by the sperm glycocalyx, which is rich in sialic acid residues [1, 2]. One of these residues, called CD52, is a highly sialated glycosylphosphatidylinositol (GPI)-anchored protein that is acquired during epididymal transit and located on the sperm plasmalemma [3–5]. During spermatogenesis, there is a massive cell-cell transfer of GPI-anchored CD52 that occurs at the sperm surface, the magnitude of which may be dependent upon the negative charge associated with the sperm plasmalemma [6]. Therefore, the presence of a negative charge may reflect normal spermatogenesis, especially since CD52 expression appears to be significantly correlated with capacitation and normal sperm morphology [3]. Consequently, this differential negative charge imparted by the sperm plasmalemma has been exploited as a means for sperm separation using either simple electrostatic [7, 8] or sophisticated electrophoretic techniques [9–11].

28.1.3 Development of Electrophoretic Technology for Sperm Sorting

This technology developed as a result of a chance contact between Professor RJ Aitken from the University of Newcastle and the CEO of Gradipore, a Sydney-based life sciences company, Tim Wawn. One of the key technical competencies developed by Gradipore was a capacity to separate biomolecules on the basis of their size and charge, as embodied in the company's core separations technology platform, Gradiflow. In collaboration with Prof Aitken's research group in gamete biology at the University of Newcastle, Gradiflow technology was harnessed to create a prototype instrument designed for sperm separation, called the cell sorter 10 (CS10). The CS10 was based upon preparative isolation by membrane electrophoresis, a patented technique that is capable of purifying most macromolecules from complex biological samples. The principle of this mode of separation was developed from the hypothesis that the CS10 preferentially selects cells on the basis of charge differences between human spermatozoa due to the differential presence of sialated proteins on the sperm plasmalemma [9]. A subsidiary commercial entity originally, SpermGen, developed the CS10 to the point that it was used in clinical trials to test its efficacy in a clinical laboratory setting (Fig. 28.1). This prototype has subsequently been reengineered by the most recent incarnation of the original Gradipore company, Memphasys Ltd., into a more sophisticated prototype, code-named Felix, for clinical evaluation by the assisted reproductive technology industry.

The fundamental instrument applies an electric potential via platinum-coated titanium mesh electrodes to move

Fig. 28.1 The CS10 instrument

spermatozoa across a 5 µm polycarbonate separation membrane, the pore size of which allows the passage of morphologically normal spermatozoa while restricting larger cells within semen, such as immature germ cells and leukocytes (Figs. 28.2 and 28.3). Spermatozoa, which are negatively charged when suspended in a physiological buffer, are attracted toward the positive electrode, or anode. Consequently, spermatozoa not possessing a normal negative charge have less electrophoretic mobility and do not manage to pass through the separation membrane during the relatively short period (<5 min) of electrophoresis. The exploitation of this concept has been found to yield a high percentage of morphologically normal, motile spermatozoa with intact DNA following electrophoretic sperm separation [9].

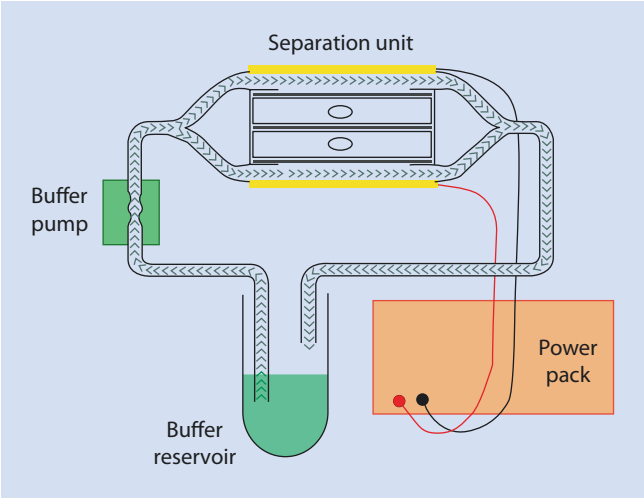


Fig. 28.2 Diagrammatic representation of the CS10 design

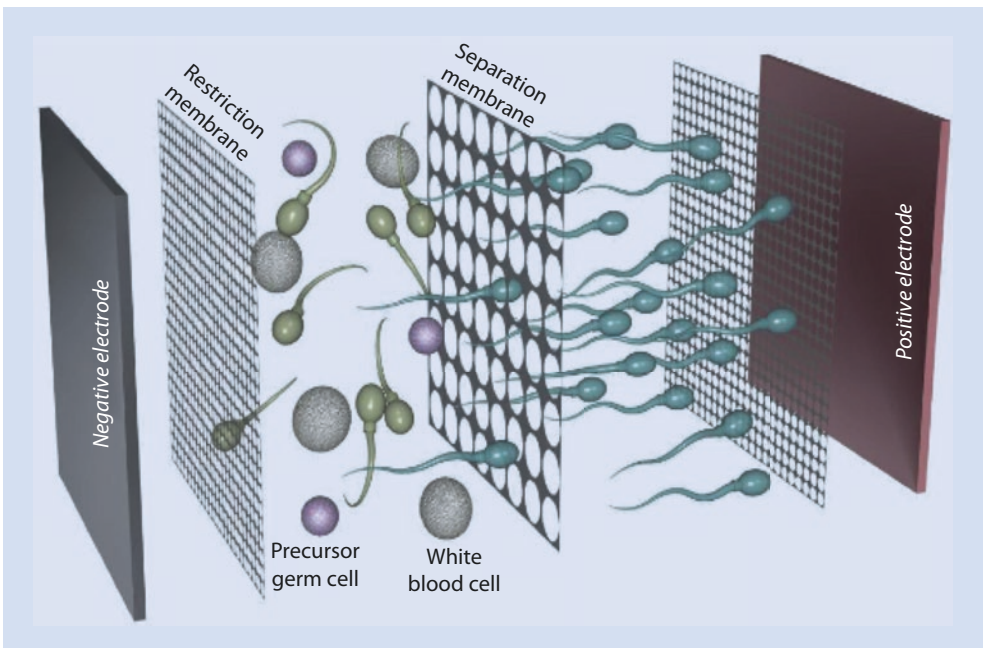
28.2 Equipment Setup and Separation Parameters

28.2.1 Separation Cartridges and Sample Handling

The separation cartridge is a self-assembled device that has an asymmetric design with an inoculation, or loading chamber volume of 2 ml, and a collection, or separation chamber volume of 400 µl (Fig. 28.4). Conveniently, 400 µl is also the estimated mean volume of the human uterine cavity and is, therefore, often the volume of sperm preparation inserted during intrauterine insemination (IUI) procedures. Consequently, the potential exists for electrophoretic sperm separation to be followed immediately by IUI of the entire volume of the sperm preparation retrieved, provided that the prostaglandins present within seminal plasma have been removed or reduced to clinically insignificant levels. However, for the purposes of in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), the spermatozoa could be used directly, provided that they are separated into an appropriate medium within the separation chamber. The component parts of the separation cartridge can be autoclaved to ensure sterility. A 5 µm polycarbonate membrane, with an active membrane area of 30 mm × 15 mm, separates the loading and separation chambers which are bounded by polyacrylamide restriction membranes with a pore size of 15 kDa that prevent cross-contamination between the semen sample and electrophoresis buffer while permitting free transit of electrolytes (Figs. 28.3 and 28.4).

The separation cartridge is inserted into the cartridge housing on top of the CS10 (Fig. 28.1), the housing being designed to ensure the cartridge can only be inserted in the correct orientation. Once the separation unit sealing mecha-

Fig. 28.3 Schematic diagram showing sperm electrophoretic mobility



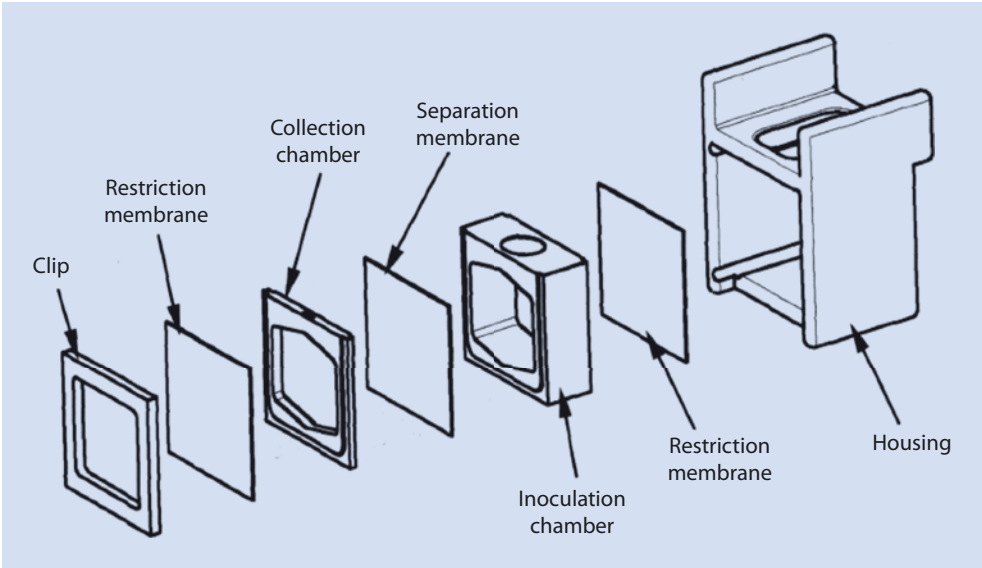


Fig. 28.4 Exploded diagram of the separation cartridge

nism is activated, the cartridge components are made watertight by the application of pressure. Semen samples are simply pipetted into the loading chamber of the cartridge using a sterile, nontoxic, disposable plastic pipette, left for 5 min to equilibrate, and then subjected to electrophoresis. Once separated, the sperm preparation is aspirated from the separation chamber of the cartridge using an elongated, sterile, nontoxic, disposable micropipette tip, as typically used in standard gel electrophoresis.

28.2.2 Electrophoresis Buffers and Temperature Settings

The electrophoresis buffer contains 10 mM HEPES, 30 mM NaCl, and 0.2 mM sucrose, having an osmolarity of 310 mOsm. kg^{-1} and a pH of 7.4, following adjustment using 2 M KOH. It is filter-sterilized prior to use with a 0.22 μm filter (Millipore Corp., Bedford, USA). In order to provide a physiological medium in which to maintain sperm viability, 400 μl of electrophoresis buffer is placed into the separation chamber prior to running a sperm separation. A sterile, disposable buffer reservoir is filled with 80 ml electrophoresis buffer and placed into the reservoir housing in front of the CS10 (Fig. 28.1). In order to prevent overheating during operation of the instrument, the buffer is maintained at 25 °C and is circulated around the instrument by means of a buffer pump (Fig. 28.2). In order to complete the electrical circuit, the buffer pump is run for at least 1 min prior to performing any sperm separations.

28.2.3 Current and Voltage Settings

The input power specifications of the CS10 are 115–240 V or 50–60 Hz. Electrophoresis is achieved via a constant current of 75 mA at a variable voltage of 18–21 V applied over a 5-min period. No electrical potential is applied until the separation run is initiated.

28.2.4 Cleaning of Equipment

At the conclusion of each sperm separation, any electrophoresis buffer remaining in the buffer reservoir is replaced with sterile, distilled water and the buffer pump is actuated to rinse the buffer lines. If no more separations are to be performed that day, the water is replaced with a 0.1 M NaOH cleaning solution, and the buffer pump is run for 30 s to circulate it through the lines of the CS10, and the cleaning solution is left in place overnight. The following morning, the cleaning solution is thoroughly rinsed out with a minimum of three washes of sterile, distilled water.

28.3 Method Validation

Initial validation of the CS10 system was performed using semen samples from normozoospermic sperm donors and a separation cartridge with a symmetrical design, the loading and separation chambers both having a capacity of 400 μl [9].

28.3.1 Sample Recovery and Purity

The mean sample concentration loaded into the system was $52 \pm 5.2 \times 10^6$ ml^{-1}. During an initial 5-min equilibration period, the starting concentration of spermatozoa in the separation chamber was $1.67 \pm 0.58 \times 10^6$ ml^{-1} (3.2% recovery), presumably as a consequence of the inherent motility of spermatozoa. Following just 30s of electrophoresis, the sperm concentration increased to $3.55 \pm 0.42 \times 10^6$ ml^{-1} (6.8% recovery), reaching a peak concentration of $22.31 \pm 5.85 \times 10^6$ ml^{-1} (42.9% recovery) after 15 min. The purity of the electrophoretically separated sperm preparations was extremely high, with contamination by round cells proving undetectable using phase-contrast microscopy [9].

28.3.2 Sperm Vitality and Motility

Sperm vitality, assessed using the eosin dye (0.05% eosin in phosphate-buffered saline) exclusion test, was 83 ± 1.5% in the original semen samples prior to electrophoresis. The percentage of viable spermatozoa in the electrophoretically separated sperm preparations was found to be consistent with that of the original samples and there was no significant change in vitality observed over the entire period (15 min) of electrophoresis [9].

Sperm motility, assessed using computer-assisted semen analysis (CASA), was 72 ± 2.1% in the original semen samples prior to electrophoresis. Similar to sperm vitality, percentage sperm motility was found to be consistent with that of the original samples and not significantly affected by the duration of electrophoresis, though a slight reduction was observed after 15 min [9]. Similarly, kinematic analysis by CASA demonstrated that the duration of electrophoresis had no significant effect upon the quality of sperm motility observed. Experiments in which field strength was progressively increased demonstrated that the sperm motility ultimately declines at higher power settings, particularly when the current was fixed and voltage was allowed to fluctuate [12]. The fact that motility is arrested under such circumstances and conditions where there is no evidence of oxidative stress or increased DNA damage suggests that exposure of human spermatozoa to an electric field might be an ideal means of achieving sperm immobilization in the context of applying ICSI therapy [12].

28.3.3 Sperm Morphology and DNA Integrity

The percentage of normal spermatozoa observed following staining by a modification of the Papanicolaou method [13] and assessed using the sperm deformity index (SDI) [14] was significantly increased ($p < 0.001$) by electrophoresis [9]. A higher percentage of morphologically normal spermatozoa within the separated sperm preparation was observed regardless of the duration of electrophoresis, with no significant variation between different time periods. Furthermore, SDI values for the separated spermatozoa were significantly below ($p < 0.001$) the threshold SDI value of 0.93 for all electrophoretic time points, indicating their normal fertilization potential [14].

DNA integrity, assessed using the terminal deoxynucleotidyl transferase-mediated deoxyuridinetriphosphate nick-end labeling (TUNEL) assay, was significantly reduced ($p < 0.05$) in the sperm preparation separated by electrophoresis [9]. This reduction was only observed at all time points up to 10 min of electrophoresis, beyond which there was no significant difference in the percentage of DNA-damaged spermatozoa.

28.3.4 Analysis of Genotype and Surface Carbohydrate Composition

Electrophoretic isolation of spermatozoa has also been found to generate sperm populations which contain approximately equal numbers of X- and Y-bearing spermatozoa after a 5-min period of separation [15]. Thus, the use of this electrophoretic sperm isolation system should have no significant impact on the gender of the progeny. In terms of the mechanisms underpinning the separation process, it is clear that the intrinsic motility of the spermatozoa is partially involved because the paralysis of sperm movement with benzoquinone does slightly reduce the number of spermatozoa isolated in this system, though not significantly [15]. However, if the surface sialic acid residues are removed from these immobilized cells with neuraminidase, then the number of spermatozoa isolated is significantly suppressed [15]. These observations confirm the overriding importance of surface sialic acid residues in carrying the negative charge that enables the electrophoretic separation process to occur.

28.4 Clinical Applications

The first successful clinical application of electrophoretic sperm separation was published as a case report following ICSI [10]. This provided proof of principle that electrophoresis could be used to prepare spermatozoa for use in assisted reproduction. However, since ICSI had been used to fertilize the oocytes in this instance, it was still unknown whether electrophoresis might compromise aspects of sperm function necessary for normal fertilization. This uncertainty was resolved following a prospective, split-sample, split-cohort controlled clinical trial, involving patients having both ICSI and IVF, with sperm prepared by either standard density gradient centrifugation (DGC) or by electrophoresis [11]. The design of this trial ensured that any differences in gamete quality between semen samples and cohorts of oocytes were controlled for. Approximately 400 oocytes were inseminated by either DGC or electrophoretically prepared spermatozoa, resulting in comparable rates of fertilization (63.6% vs 62.4%, respectively), cleavage (88.5% vs 99.0%, respectively), and embryo quality (26.1% vs 27.4% top grade embryos, respectively), regardless of whether ICSI or IVF was employed as the method of insemination [11]. Furthermore, six pregnancies resulted from the use of electrophoretically prepared spermatozoa, two of them from patients receiving ICSI and four from patients receiving IVF [11].

Previous work has demonstrated that spermatozoa can be efficiently isolated from a variety of sources [10]. Separation of frozen-thawed, cryostored semen ($39.6 \pm 11.1 \times 10^6$ ml^{-1}) resulted in 27% recovery of separated spermatozoa ($10.8 \pm 3.8 \times 10^6$ ml^{-1}) after just 5-min electrophoresis [10]. These sperm preparations were devoid of detectable contaminating cells, the separated spermatozoa displaying significantly greater viability ($p < 0.01$), motility ($p < 0.05$), and normal morphology ($p < 0.001$) than the cryostored semen [10]. Therefore, electrophoresis may prove an advantageous method for preparing cryostored semen, especially since it has recently been shown that such material is particularly

vulnerable to oxidative stress and subsequent DNA damage during processing by standard DGC [16].

A particularly promising potential application of electrophoretic sperm separation is the isolation of spermatozoa exhibiting low levels of sperm DNA damage from more complex mixtures of cells such as those found in surgically recovered aspirates and biopsies of the epididymis and testis. Testicular biopsy material, containing a range of mature and immature spermatozoa, has been shown to rapidly yield cells with greater residual motility, vitality, and normal morphology than those in the original biopsy following electrophoretic sperm preparation [10]. Importantly, the recovery of spermatozoa from the biopsy material was good (28.4 ± 7.1%).

28.5 Closing Remarks

Combined together, the basic scientific and clinical data suggest that electrophoretic sperm separation is particularly suitable for those patients requiring ICSI or IVF where the cause of infertility is due to poor sperm morphology and/or significantly damaged sperm DNA. Though electrophoresis has previously been demonstrated to be detrimental to sperm motility in a free-flow electrophoretic system [17], such impacts on sperm quality do not appear to be a problem with the system described above. The latter would therefore seem to offer some promise as a fast, efficient method for isolating spermatozoa exhibiting low levels of DNA damage for assisted conception applications ranging from IUI to ICSI [11].

Review Questions

1. What are the major approaches used to isolate spermatozoa in the context of assisted reproductive technology (ART)?
2. What are the basic principles underpinning the electrophoretic isolation of spermatozoa?
3. What are the key attributes of the sperm subpopulations isolated with this technique?
4. How has this device performed to date in a clinical setting?
5. Does it represent the future of sperm isolation in an ART context?

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Magnetic Activated Cell Sorting of Human Spermatozoa

Enver Kerem Dirican

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Learning Objectives

- Sperm selection strategies in assisted reproduction
- Apoptosis in human spermatozoa
- Basic principles of magnetic cell sorting
- Improvement of assisted reproduction outcome

29.1 Introduction

Magnetic separation has been successfully applied to many aspects of both biomedical and biological research and also in clinical areas like cellular therapies [1] for human autoimmune diseases, like rheumatoid arthritis [2], diabetes [3], multiple sclerosis [4], and systemic lupus erythematosus [5], and nucleic acid transfer as a transfection method [6] to optimize conditions for virus-mediated gene delivery (therapy) by magnetofection [7].

In the last decade, several studies have been carried out on the use of magnetic cell sorting in human reproduction, for decontamination of testicular cell suspensions in cancer patients [8] and for elimination of apoptotic spermatozoa from human semen samples [9–11].

Infertile men with poor sperm motility and morphology were found to have increased sperm DNA fragmentation compared with individuals with normal semen parameters [12]. Men with normal semen analysis may also have a high degree of sperm DNA fragmentation, which can be a major cause of unexplained infertility, and sperm DNA fragmentation may result from aberrant chromatin packaging during spermatogenesis [13], defective apoptosis before ejaculation [14], or excessive production of reactive oxygen species (ROS) in the ejaculate [15]. Exposures to environmental or industrial toxins [16], genetics [17], and lifestyle [18] are also known factors that may cause sperm DNA fragmentation and infertility.

Although the factors present in the paternal genome that may have an impact on poor reproductive outcome are still not well defined, there is accumulating evidence linking sperm nuclear DNA abnormalities to poor reproductive outcome and one of the most suspected organelles is the sperm nucleus. Studies reveal that severe teratozoospermia results in high preimplantation embryo aneuploidy [19, 20] and the interchromosomal effect is related to impaired semen parameters [21]. Studies have also shown that immature sperm have increased rates of lipid peroxidation and bear poor morphometric and morphological attributes, zona pellucida-binding properties, and fertility [22]. The ROS-induced lipid peroxidation is involved in the mechanisms by which spermatozoa are damaged in many cases of male infertility. Studies show a significant correlation between sperm morphology attributes and the expressed apoptotic markers like caspase-3 activation and mitochondrial membrane potential integrity [23].

The sperm nucleus, as the carrier of paternal DNA to the oocyte, remains as the greatest contributor to the potential success of reproductive outcome, where sperm nuclear DNA strand breaks, DNA repair mechanisms, apoptosis, and DNA remodeling processes are the main factors to be considered [24].

A number of novel sperm preparation and selection techniques have now been proposed that may assist in limiting the chance of selecting an abnormal spermatozoon prior to intracytoplasmic sperm injection (ICSI) [25]. In terms of preparing the whole sperm sample and enrichment of high-quality spermatozoa, a novel microfluidic system has been introduced [26]. The use of high-magnification microscopes to identify minor morphological defects of the living spermatozoa [27], selection of mature spermatozoa referring to the binding ability of human spermatozoa to hyaluronic acid [28], and a selection based on sperm membrane charge by a microelectrophoretic method [29] are also mentioned.

Programmed cell death in animals usually occurs by apoptosis. Cells dying by apoptosis undergo characteristic morphological changes. Most importantly, the surface of the cell becomes chemically altered, so that a neighboring cell or a macrophage rapidly engulfs them, before they can spill their contents [30].

An especially important change occurs in the plasma membrane of apoptotic cells. The negatively charged phospholipid phosphatidylserine (PS) is normally exclusively located in the inner leaflet of the lipid bilayer of the plasma membrane, but it flips to the outer leaflet in apoptotic cells by the activity of the enzyme scramblase, where it can serve as a marker of these cells. This process blocks the inflammation associated with phagocytosis. The PS on the surface of apoptotic cells can be visualized with a labeled form of Annexin V protein, which specifically binds to this phospholipid [30].

29.2 The Technique

The Annexin V-coated microbeads are used for the isolation of cells with exposed PS and removal of dead and apoptotic spermatozoa from seminal samples. Briefly, the PS-exposing spermatozoa are magnetically labeled by the protein Annexin V; then the magnetically labeled apoptotic and dead spermatozoa are retained on a magnetic activated cell sorting (MACS) column where the unlabeled non-apoptotic spermatozoa are in the flow-through. At the end, magnetically labeled, PS-exposing spermatozoa can be eluted from the column for various cellular tests (Miltenyi Biotec GmbH, Germany) [31].

The MACS technology is inexpensive and easy to perform. A brief step-by-step protocol is as follows:

- Perform all steps under a laminar air flow hood using aseptic techniques. Use only cell culture-tested disposable materials.
- Allow ejaculate to liquefy and evaluate the seminal parameters.
- Prepare 1 × Binding Buffer from 20 × Stock Solution (Miltenyi Biotec GmbH, Germany).

- Centrifuge sperm cells for 10 min at 300 × g. Remove supernatant and resuspend sperm pellet in 80 µl of 1 × Binding Buffer per 10^7 total sperm count.
- Add 20 µl of MACS Annexin V MicroBeads (Miltenyi Biotec GmbH, Germany) per 10^7 total sperm count, mix gently, and incubate for 15 min at 6°–12 °C, preferably in a temperature-controlled laboratory refrigerator.
- Wash sperm cells by adding 2 ml of 1 × Binding Buffer, centrifuge at 300 × g for 10 min, remove supernatant completely, and resuspend sperm pellet in 500 µl of 1 × Binding Buffer.
- For magnetic sperm separation, use a MS Column (Miltenyi Biotec GmbH, Germany). Place the column in the MACS separator (Miltenyi Biotec GmbH, Germany).
- Prepare column by washing with 500 µl volume of 1 × Binding Buffer.
- Apply sperm suspension in 500 µl amount of 1 × Binding Buffer onto the column. Let the cell suspension pass through drop by drop, and then rinse the column with 500 µl of 1 × Binding Buffer 4 times. Collect the sperm suspension and 2 ml Binding Buffer in the same test tube.
- Evaluate the post-separation sperm values.
- Either perform a density gradient centrifugation or a swim-up preparation on the sperm suspension according to sperm concentration and motility.
- Use the prepared motile sperm cells for intracytoplasmic sperm injection (Fig. 29.1).

29.3 Basic Studies

Several studies using MACS technique with human spermatozoa have been published over the years. Interests in these studies were mainly the molecular efficiency of the technique and improving the post-preparation sperm quality. One of the first studies on the yield of magnetic selection in human spermatozoa came in the mid 2000s. Researchers have evaluated the percentage of sperm recovery following the use of MACS as a sperm preparation technique, and they concluded that the integration of MACS with density gradient centrifugation (DGC) is an effective sperm preparation technique that does not lead to significant cell loss and separating a distinctive population of non-apoptotic spermatozoa with intact membranes might optimize the outcome of assisted reproduction [11]. Later on, the separation effect of MACS on capacitation and acrosome reaction was investigated in the non-apoptotic sperm fractions. Non-apoptotic human spermatozoa with intact plasma membranes were found to be characterized by a superior ability to capacitate and consequently by maximum potential to perform acrosome reaction after stimulation [32].

Studies have also shown that non-apoptotic sperm fractions selected by MACS technique have morphologically superior quality [23]; higher percentage of motility, viability, and apoptosis indices [33]; and routine sperm parameters [34].

At the end of the 1990s, separation of leucocytes from human seminal plasma was successfully performed by magnetic selection [35]. Later on, in the early 2000s, scientists focused on studying molecular analyses on human spermatozoa before and after MACS selection. It was shown that spermatozoa with deteriorated membrane and exposed PS are characterized by an increased lyso-phosphatidylcholine content that is likely generated by phospholipases [36] and also are characterized by an increase in activated caspases, which were found in infertile patients [9].

Reduction of apoptotic spermatozoa within the ejaculate by means of the MACS system results in a distinct reduction of spermatozoa with DNA fragmentation [37], enrichment of spermatozoa free of apoptosis [38], improvement of sperm viability, motility, and mitochondrial membrane integrity [39], elimination of aneuploidy [40], and enrichment of spermatozoa with good chromatin quality for both normozoospermic and also asthenoteratozoospermic seminal values [41].

The first report on the initiator and effector caspases of the main pathways of apoptosis in ejaculated human spermatozoa came in the early 2000s, and in that study, activated caspases were found especially in spermatozoa with disturbed membranes, where cryopreservation was used as a tool for increasing the number of spermatozoa showing an activation of caspases [42]. Further studies were performed on human sperm cryopreservation and the benefit of MACS on eliminating apoptotic sperm fractions from frozen-thawed semen samples. Studies revealed out that MACS separation before cryopreservation results in depletion of spermatozoa which are positive for activated caspases [10], depletion of low-quality spermatozoa from cryopreserved semen samples [43], and selection of a population of non-apoptotic spermatozoa which optimizes cryopreservation and thawing outcome [44].

29.4 Clinical Use

The integrity of the paternal genome is of paramount importance in the initiation and maintenance of a viable pregnancy in both natural and assisted reproduction. Studies have investigated the sperm's fertilizing potential using hamster oocyte penetration assay [45], and results suggested that non-apoptotic spermatozoa prepared by MACS display higher fertilization potential following ICSI, and this technique should be evaluated in a clinical setting for its impact on ICSI outcomes [46]. Studies also showed a reduced level of apoptotic markers, improved acrosome reaction scores [47], and superior morphological quality [48] after MACS preparation which may contribute to increased implantation and pregnancy rates.

The first assisted reproduction data and clinical pregnancies in the world with the use of MACS as a sperm preparation method in human assisted reproduction were presented in 2006 in a local meeting [49] as a preliminary study. MACS preparation did not yield any statistically significant improvement in terms of fertilization and embryo cleavage rates and embryo quality. This was related to the limited number of cases by the authors.

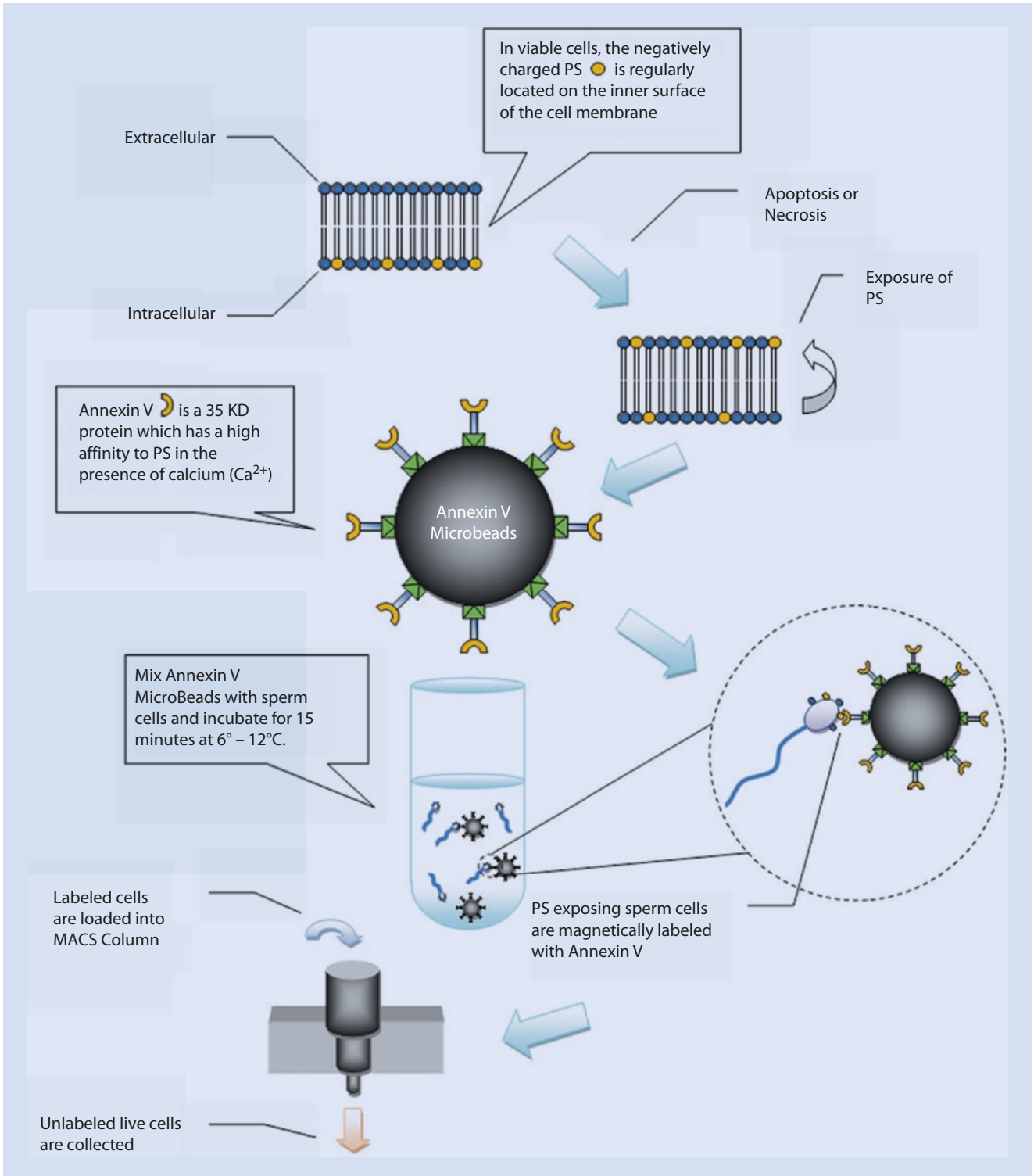

Fig. 29.1 The MACS technology is inexpensive and easy to perform. A brief step-by-step protocol is as follows

In 2008, a prospective study was published to assess the impact of MACS technique for selection of non-apoptotic spermatozoa on the outcome of ICSI [31]. The study compared the cleavage, fertilization, implantation, and pregnancy rates associated with two sperm preparation methods, MACS and DGC, in male factor cases. Although there were no significant differences between the two groups in terms of fertilization rates and embryo quality, there was a statistically significant improvement in the cleavage rate of the MACS group embryos. This difference resulted in a significantly

higher number of embryos on the embryo transfer day, by reducing the number of arrested embryos during in vitro culture [43]. Early embryo arrest is associated with possible deleterious factors [50–52] that can be introduced into the human oocyte by spermatozoa [53, 54] and impair the assisted reproduction outcome.

Another study reported 3 ongoing clinical pregnancies out of 10 cases by using MACS as a sperm preparation technique [55]. They also represented their first healthy baby born after MACS technique [56].

In 2013, healthy live births were reported after the use of MACS in cryopreserved spermatozoa [57]. A 4-year-old boy born after MACS-ICSI-TESA was examined for psychological and somatic scores and was found healthy in 2015 [58]. A unicentric, prospective, and randomized study was reported in 2015 on the outcome of MACS and hyaluronic acid binding, where MACS yielded higher clinical pregnancy rates [59]. But on the other hand, a Cochrane Review did not find any evidence on the effects of MACS on assisted reproduction outcome [60]. In order to address the safety issues, obstetric and perinatal outcome of babies born from sperm selected by MACS were examined and it was concluded that MACS technology did not increase or decrease adverse obstetric and perinatal outcomes [61].

A number of studies have shown that using spermatozoa prepared with MACS technique significantly improves the quality of post-preparation spermatozoa. These studies indicate that MACS enriches the sperm population by eliminating apoptotic, necrotic, chromosomally abnormal, and DNA-fragmented spermatozoa [38–40]. A systematic review was designed to evaluate the efficacy of MACS on assisted reproductive technology (ART) success rates and concluded that it appears to be safe and efficient to select spermatozoa [62]. On the other hand, a controlled and randomized trial implied that in an unselected male population, MACS did not have any beneficial effect on ART outcome both in terms of embryo quality and also pregnancy rates [63]. Unfortunately, there is no clear evidence that MACS technique has improved implantation and live birth rates in large clinical trials. Recently, a good manufacturing practices (GMP) product was introduced in the market which will be useful to put the MACS technique into clinical use in assisted reproduction.

Review Questions

1. What is the purpose of eliminating apoptotic spermatozoa prior to assisted reproduction?
2. Explain the depletion and enrichment techniques in magnetic cell sorting technologies.
3. What are the advantages of sperm selection in terms of improving assisted reproduction outcome?

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Insemination Procedures

Contents

Intrauterine Insemination

Gautam N. Allahbadia, Rubina Merchant, Akanksha Allahbadia, Goral Gandhi, and Swati Allahbadia

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Learning Objectives

Upon reading this chapter, the practitioner will be able to:

- Learn the indications for IUI.
- Learn the good medical practice-based workup of the infertile couple for IUI.
- Have an overview about the correct ovarian stimulation protocols for IUI.
- Know the correct techniques of sperm preparation for IUI.
- Physicians will learn the finer nuances of the correct technique of IUI.
- Learners will know how to overcome difficult IUIs and learn how to develop motor and cognitive skills for performing "ideal" IUIs.
- The goal is for practitioners to have an updated knowledge about the subject with recent advances in the field.

30.1 Introduction

Despite revolutionary advances in the field of assisted reproduction, such as in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), and subzonal insemination (SUZI), intrauterine insemination (IUI) remains an inexpensive, non-invasive, and effective first-line therapy for selected patients with cervical factor, moderate male factor, unexplained infertility, immunological infertility, and infertility due to ejaculatory disorders and is now also proposed as a therapy for endometriosis, ovarian dysfunction, and even for tubal factor infertility. Though the technique of IUI has essentially remained the same, several advances in the type of stimulation protocols, gonadotropins, sperm preparation techniques, and ultrasound monitoring have led to promising success rates with IUI. IUI involves a pre-procedure evaluation including a thorough physical, clinical, and laboratory evaluation to determine the duration and etiology of infertility, semen quality, functional tubal status, and the follicular and uterine status. Controlled ovarian hyperstimulation with close monitoring of folliculogenesis and ovulation to avoid adverse complications, such as ovarian hyperstimulation syndrome (OHSS) and multiple pregnancies, may be used to obtain the adequate number of follicles. Following induction of ovulation with human chorionic gonadotropin (hCG), the processed semen sample is then inseminated in the uterine cavity. The objective in terms of preovulatory follicle number must be determined prior to stimulation in order to optimize the cycle outcome with a singleton birth [1]. IUI is the preferred conception-enhancing technique for women <35 years, functional tubes, short period of infertility, and moderate male infertility, particularly in technology-limited settings, and four to six IUI cycles may be performed before considering alternate therapy such as IVF [2, 3]. It is the method of choice vs. timed intercourse or natural cycle IUI [1]. The following sections will deal with the indications for IUI, technique, stimulation protocols, alternative insemination techniques, and prognostic factors that determine the outcome.

30.2 Indications for IUI

30.2.1 Cervical Factor Infertility

In couples with a cervical factor, diagnosed by a well-timed, non-progressive post-coital test with normal semen parameters [4], higher pregnancy rates (PRs) have been reported following IUI compared to expectant management (51% vs. 33%, respectively) [5] with acceptable pregnancy rates even without COH (9.7%) and without an increased risk for multiple pregnancy compared to COH (12.7%) [4]. Cumulative pregnancy rates of 19.7%, 36.8%, and 36.8% have been reported for a maximum of three IUI cycles in patients with a cervical factor without superovulation [6].

30.2.2 Male Factor Infertility

A male factor may be diagnosed if the semen analysis at an initial microscopic examination demonstrates low sperm count, motility, morphology, antisperm antibodies, or any combination of these or advanced testing indicates subnormal sperm function. While IUI is considered the best first-line treatment and most cost-effective procedure for moderate male factor subfertility [7], severe male factor infertility with a significant deterioration in sperm parameters or function may necessitate a direct referral to IVF or ICSI in that order, depending on the degree of severity [8]. Pregnancy rates of 12.8%, 29.3%, and 38.3% for a maximum of three cycles have been reported in couples with a male factor without superovulation [6], 7% per cycle following COH-IUI with clomiphene citrate (CC), and 12% per cycle with follicle-stimulating hormone (FSH) with multiple birth rates averaging 13% [9].

30.2.3 Unexplained Infertility

Despite the belief that IVF may be a more cost-effective primary treatment option compared to IUI in lieu of the low success rates with IUI and the subsequent requirement for IVF in the event of failure [10], the results of randomized controlled trials (RCTs) using live birth rates rather than pregnancy rates, and taking into account efficacy, complications, especially multiple pregnancy rates, patient compliance, and cost-efficiency, suggest that the initial treatment for idiopathic infertility should be IUI as opposed to IVF [11]. Although the pregnancy rate per cycle was higher in the IVF group than in the IUI groups [12.2% vs. 7.4% (spontaneous cycle) and 8.7% (COH-IUI), respectively; $p = 0.09$], the cumulative pregnancy rate for IVF was not significantly better than that for IUI. Moreover, the drop-out rates before a maximum of six attempts were higher in the IVF group compared to the IUI groups (42% vs. 15% and 16%, respectively; $p < 0.01$) [12].

30.2.4 Tubal Factor

Lower cumulative PRs have been reported in couples with isolated unilateral tubal occlusion compared to unexplained infertility after three cycles of COH-IUI (30.9% vs. 42.6%); cumulative PRs were lower in women with mid-distal or distal tubal occlusion compared to women with proximal tubal occlusion (19% vs. 38.2%) and significantly lower than in women with unexplained infertility (19% vs. 42.6%, respectively). Hence, though COH-IUI can be suggested as the initial treatment of choice in patients with unilateral proximal tubal occlusion, patients with mid-distal or distal tubal occlusion on hysterosalpingography (HSG) should be referred for laparoscopic assessment or IVF [13].

30.2.5 Endometriosis

An estrogen-dependent disorder, defined as the presence of endometrial tissue outside of the uterine cavity, endometriosis is a leading cause of infertility with a prevalence of 0.5–5% in fertile and 25–40% in infertile women [14]. Controlled ovarian hyperstimulation with IUI is recommended in early-stage and surgically corrected endometriosis when the pelvic anatomy is normal, while combined surgery with gonadotropin-releasing hormone (GnRH) analogue treatment has been proposed to be a first-line therapy followed by IVF as second-line therapy in advanced cases [15]. Comparable clinical PRs per cycle have been reported in women with minimal, mild endometriosis and unexplained infertility (21% vs. 18.9% vs. 20.5%) following COH-IUI with comparative cumulative live birth rates within four cycles of COH and IUI (70.2%, 68.2%, 66.5%, respectively); CPR/cycle with or without COH-IUI was lower in women with surgically untreated minimal to mild endometriosis than in women with unexplained infertility [16]. However, in patients with minimal or mild endometriosis with pathological uterotubal transport documented by hysterosalpingoscintigraphy (HSSG), IUI yields poor pregnancy rates despite normal semen parameters and patent fallopian tubes, necessitating recourse to IVF/ICSI [17]. Though IVF reduces time to pregnancy in early-stage disease compared to controls, it does not increase the chance of pregnancy after 3 years [18]. In patients with stage IV endometriosis and in women >38 years of age, significantly higher PR, fecundity, and cumulative fecundity have been reported following in vitro fertilization-embryo transfer (IVF-ET) compared to COH-IUI. Hence, IVF-ET should be the first-line approach in the management of infertility in such patients, and if COH-IUI is attempted, it should not exceed three to four cycles [19].

30.2.6 Contraindications

IUI may be contraindicated in women with sperm-immobilizing antibodies owing to antibodies secreted in the female reproductive tract that might impair sperm passage, inhibit fertilization, and prevent normal postfertilization processes [20]. Couples with severe male factor infertility, abnormal pelvic anatomy, not amenable to microsurgical repair; fallopian tube disease with an impaired ampullary mucosal architecture; advanced stage endometriosis (stages III and IV); or minimal/mild endometriosis (stages I and II) with documented failure in tubal transport may directly benefit from IVF/ICSI with or without surgical interventions. The total antral follicle number is reported to decrease with age ($P < 0.0001$). In women >35 years with antral follicle counts (AFCs) <5, the application of COH/IUI may not be indicated [21].

30.2.7 Workup Before IUI

Major causes of infertility include male factors, ovarian dysfunction, tubal disease, endometriosis, and uterine or cervical factors. A careful history and physical examination of each partner can reveal the underlying etiology and guide further management of infertility.

Investigations that must be carried out in the female partner include:

- Evaluation of folliculogenesis and documentation of ovulation by transvaginal sonography (TVS) or with a home urinary luteinizing hormone kit.
- Evaluation of the functional tubal status by HSG, confirmed by a laparo-hysteroscopy in case of an abnormality, which has diagnostic and therapeutic power and may guide future management.
- Further, a HSSG in patients with minimal or mild endometriosis may help document pathological uterotubal transport characteristic of the disease, necessitating IVF/ICSI despite normal semen parameters [17].
- A complete hormone evaluation including FSH, luteinizing hormone (LH), and thyroid hormone profile.
- Ovarian reserve testing of FSH and estradiol (E2) levels on day 3 of the menstrual cycle, the clomiphene citrate challenge test, or pelvic ultrasonography for AFC in women >35 years to determine treatment options and the likelihood of success [22].
- Testing for antisperm antibodies (ASAB) in the serum and cervical mucus.
- Infections such as chlamydial salpingitis, genital tract tuberculosis, and those caused by *Chlamydia trachomatis* and *Neisseria gonorrhoeae*.
- Testing for HIV/hepatitis.

30.2.8 Male Partner

- A thorough semen analysis. Abnormal seminograms of infertile men may be classified as azoospermia (no sperm in the ejaculate), oligozoospermia (sperm count <15 million/mL), cryptozoospermia (spermatozoa absent from fresh preparations but observed in a centrifuged pellet), teratozoospermia (normal sperm

morphology <4%), asthenozoospermia (progressive motility <32%), necrozoospermia (>58% dead sperm), or a combination of any of these conditions.
- Sperm function tests to evaluate sperm survival, viability, acrosome reaction, and sperm fertilizing ability.
- Testing for antisperm antibodies.
- Semen culture and sensitivity to evaluate infection.
- Testing for HIV/hepatitis.

30.3 Natural Cycles or Controlled Ovarian Hyperstimulation?

IUI may be performed with or without controlled ovarian hyperstimulation depending on the patient characteristics.

30.3.1 Natural Cycles

A majority of studies have reported better IUI outcomes in terms of the number of preovulatory follicles, clinical and ongoing pregnancy rates (OPRs), and live birth rates following ovulation induction (OI) compared with the natural cycle [23–28]. Significantly higher pregnancy rates have been reported following ovulation induction with CC/CC + gonadotropins/gonadotropins alone compared to natural cycles in husband (AIH) inseminations [26, 27] (Table 30.1), while no significant differences were observed between natural cycles and donor insemination (DI) cycles [27]. The overall pregnancy rates for AIH and DI cycles were 8.6% and 20.2%, respectively [27]. Pittrof et al. [23] reported a significantly higher number of preovulatory follicles (43.6%, 59.9%, 12.6%, $P < 0.0001$) and significantly higher pregnancy rates ($P = 0.038$) in CC/tamoxifen + gonadotropin-stimulated cycles compared to natural cycles [23]. However, Chen and Liu [26] concluded that though stimulated IUI is superior to natural cycle IUI in patients <35 years, natural cycle is preferable for patients ≥35 years. There were no significant differences in the abortion and delivery rates between the OI and the natural cycle insemination ($P > 0.05$) [26]. IUI in the spontaneous cycle carries fewer health risks than does IUI after mild hormonal stimulation and, therefore, should be the first-choice treatment [12].

30.3.2 Controlled Ovarian Hyperstimulation

Controlled ovarian hyperstimulation (COH) can be used in conjunction with IUI in couples with infertility factors not involving the fallopian tubes to increase the number of available oocytes at the site of fertilization [29, 30]. Ovarian stimulation is initiated after the selection of the dominant follicle, mostly on the fifth day of the cycle. Several drugs, including clomiphene citrate (CC), tamoxifen, aromatase inhibitors, and gonadotropins alone or in conjunction with GnRH agonist/antagonist, have been used for ovulation induction in various protocols that may be individually tailored according to the patient. These regimens and protocols and clinical evidence of their benefits are discussed in the following sections.

30.3.3 Clomiphene Citrate

Ovarian stimulation by clomiphene citrate (CC) and IUI remains the first-choice treatment for ovulatory dysfunction, unexplained infertility, endometriosis, or male subfertility [27] with pregnancy rates averaging 7% per cycle [8]. Clomiphene citrate, an antiestrogenic agent, is often administered for 5 days from day 3 to 7 of the menstrual cycle in doses of 50 mg once/twice a day. However, pelvic abnormalities, especially ovarian cysts, must be excluded prior to CC administration. Owing to the negative influence of CC on endometrial thickness, the medication should be reduced to 3 days in patients with retarded endometrial growth confirmed by ultrasonography [31]. Though no consensus exists about the drug of first choice to be used as hyperstimulation and there are no significant differences in clinical pregnancy (38% vs. 34.3%) and live birth rates (28.2% vs. 26.9%) between CC and recombinant FSH (rFSH), a randomized multicenter parallel trial concluded that being less expensive, CC seems to be the more cost-effective drug and, therefore, can be offered as drug of first choice [32].

30.3.4 Gonadotropins

Gonadotropins, such as FSH and human menopausal gonadotropin (hMG), alone or in conjunction with GnRH agonist/antagonist, are often used for ovulation induction. The dose and mode of administration of gonadotropins are adjusted according to the individual patient and may be varied depending on the response. The response of the patient to gonadotropins is monitored regularly by TVS. A meta-analysis of 43 trials involving 3957 women concluded that gonadotropins might be the most effective drugs when IUI is combined with ovarian hyperstimulation, yielding higher pregnancy rates compared to antiestrogens, comparable PRs with different types of gonadotropins, no improvement with GnRH agonist or antagonist but increased multiple pregnancy rates and OHSS rates with increased doses of gonadotropins, and significantly higher multiple pregnancy rates with the agonist. When gonadotropins are used for ovarian stimulation, low-dose protocols are advised since pregnancy rates do not differ from those obtained with high-dose regimens, whereas the chances to encounter negative effects from ovarian stimulation such as multiple pregnancies and OHSS are limited with low-dose gonadotropins. Further research is needed for each comparison made [33]. No significant differences have been reported among low-dose gonadotropin proto-

Table 30.1 Comparison in the clinical pregnancy rates following stimulation with various regimens and IUI

Study	Natural cycles	Stimulation regimens								*P* value
		CC	CC + gonadotropins		Tx + G	Aromatase inhibitors		Gonadotropins		
			hMG	rFSH		Letrozole	L + rFSH	hMG	rFSH	
Baysoy et al. [41]						18.4		15.7[a]		
Tehrani Nejad et al. [40]			14.3			32.8				<0.01
Gregoriou et al. [45]						8.9			14.0	
Barroso et al. [38]				20			23.8[b]			
Mitwally and Casper [43]							26.5		18.5	
Bedaiwy et al. [42]							15.77		18.07	
Mahani and Afnan [25]	2.0 (12.0)	4.0 (7.0)	7.0 (16.0)					9.0 (23.0)		
Pittrof et al. [23]	20.1		23.6		29.9					0.038
Chen and Liu [26]	11.35		19.61							<0.01
Custers et al. [27]	6.3	12.5	16.4					11.2		

CC was administered at a dose of 100 mg/day from day 3 to 7 of the menstrual cycle. Letrozole was administered at a dose of 5 mg/day from day 3 to 7 of the menstrual cycle unless otherwise specified. Gonadotropins were administered at a dose of 75 IU from day 6 to 7 of the menstrual cycle until hCG administration

Values in brackets represent CPR/patient

Tx + G tamoxifen + gonadotropins

[a]hMG was administered on day 3 at a dose of 75 IU for women <30 years old and 150 IU for women >30 years old

[b]Letrozole was administered at a dose of 2.5 mg/day from day 3 to 7 of the menstrual cycle

cols that differed in gonadotropin dosage (4/6/8/ampoules of 75 IU FSH) or the mode of administration in terms of cycle parameters, suggesting that an individualized and more intensive approach to ovarian stimulation is necessary for many women with unexplained infertility [34]. Lyophilized urinary hMG (HP-hMG) and rFSH are equally suitable in mild ovarian stimulation for IUI in patients with unexplained infertility. However, data derived from a larger study population are needed to determine whether higher amounts of two gonadotropins in this subgroup might produce any benefits or unfavorable effects [35].

With regard to the mode of administration, daily recombinant FSH (follitropin-beta) stimulation has been associated with higher CPRs (42% vs. 19%, respectively), higher total recombinant FSH dose (825 IU vs. 625 IU), and endometrial thickness (10.1 mm vs. 9.3 mm) compared to alternate-day FSH stimulation in women with anovulatory or unexplained infertility for over 12 months who had not responded to or not conceived with CC treatment though the duration of stimulation and the median number of follicles over 14 mm, AFC, and day 3 serum FSH were comparable between the groups. However, prospective randomized trials would be needed to determine whether this is indeed the case [36]. Mahani and Afnan [25] reported the highest CPRs/cycle and CPR/patient following IUI in patients stimulated with hMG compared with CC, CC + hMG, or natural cycle.

30.3.5 Combination Protocols

Clomiphene citrate may be used in conjunction with gonadotropins/dexamethasone for stimulation. Combination protocols are less costly and equally effective, with potentially fewer multiple births than with gonadotropins alone with fewer days of injections and fewer ampoules used. The advantages of a combination protocol in terms of pregnancy rates are depicted in Table 30.1. Although tamoxifen (40 mg/day from day 3 to 9) may not be a first-line treatment in patients with adequate endometrium, it may be a promising alternative for patients with a thin endometrium, yielding increased endometrial thickness ($P < 0.001$), CPR ($P = 0.015$), decreased early miscarriage rate ($P = 0.001$), and, thus, improved ongoing pregnancy rate ($P < 0.001$) compared to CC (100 mg/day for 5 days), despite a higher gonadotropin dose and stimulation duration and fewer follicles larger than 14 mm than clomiphene-treated patients [37].

30.3.6 Aromatase Inhibitors

Studies have reported a beneficial effect of the use of the aromatase inhibitor letrozole (2.5–5 mg/day from day 3 to 7 of the IUI cycle) alone/coadministered with gonadotropins compared to CC, CC + gonadotropins, or gonadotropins alone in terms of comparable if not higher CPR/cycle and take-home

baby rates. Significantly higher serum levels of LH, endometrial thickness, and progesterone at the time of hCG administration have been observed despite a significantly lower serum E2 level [37–39] with significantly lower costs, risks, and patient inconvenience in patients with unexplained infertility [37, 38, 40, 41], endometriosis, and combined indications [42] and lower FSH dose requirement and IUI cancelation rates in patients with ovulatory infertility [43] and older infertile women [39]. Letrozole may be more effective than clomiphene and tamoxifen in a combination protocol [44] and beneficial in patients who fail to respond to CC [45] (Table 30.1). The improved follicular response to aromatase inhibitors has been attributed to aromatase inhibition, a release in the estrogenic negative feedback, increased endogenous FSH secretion, and increased follicular sensitivity to FSH [43].

30.3.7 Additives

Abdel Razik's study evaluated the effects of nitric oxide donor's treatment on the pregnancy rate and uterine blood flow in patients with unexplained infertility undergoing clomiphene citrate stimulation and intrauterine insemination [46]. A total of 120 patients were randomly allocated to a control group who received 100 mg clomiphene citrate daily from day 5 to 9 of cycle plus placebo vaginal tablets, and a study group received clomiphene citrate plus isosorbide mononitrate 10-mg vaginal tablets. Vaginal ultrasound was done before treatment and every other day starting from day 12 of cycle to count mature follicles, and ovulation was triggered by IM injection of 10,000 IU hCG when one follicle measured ≥ 18 mm followed by intrauterine insemination after 36 h. The endometrial thickness, uterine arteries' resistance and pulsation indices, and endometrial vascular flow and vascular flow indices were measured before treatment and at the day of hCG injection. Results were analyzed after one cycle treatment using the mean ± SD, the student t test, and the Fisher Exact test. Significant result was considered at p values < 0.05. The study group had significant higher pregnancy rate/cycle and higher endometrial and lower uterine artery blood flow indices ($p < 0.05$) [46].

30.3.8 Stimulation Protocols

Various gonadotropin stimulation protocols have been employed depending on the patient characteristics. Stimulation may be initiated on the first day of a cycle (the short protocol) or in the cycle preceding the "proper" stimulation cycle (the long protocol).

30.3.9 The Short GnRH Analogue Protocol

GnRH analogue (0.1 mg Decapeptyl/triptorelin) is administered daily from the first day of a cycle until hCG administration, resulting in a gonadotropin flare-up effect. Gonadotropin administration (hMG/FSH) is initiated from the third day of the cycle in a step-up/step-down protocol, doses being adjusted individually according to patient requirements.

30.3.10 Step-Up Protocol

Generally, hMG is administered in a daily dose of 150 IU that may be stepped up to 225 IU when one or more follicles are ≥14 mm and the dose maintained until hCG administration. Low-dose step-up FSH treatment has been associated with a significant reduction in the incidence of OHSS compared to the conventional dose (8.3% vs. 27.1%, $P < 0.05$) and a significant decrease in the incidence of moderate OHSS requiring hospitalization (0% vs. 16.7%, $P < 0.01$) in patients with unexplained infertility despite comparable pregnancy rates. However, multiple pregnancies cannot be completely prevented with the low-dose protocol [47].

30.3.11 Step-Down Protocol

Gonadotropins (hMG) are administered in a daily dose of 225 IU that may be stepped down to 150 IU if an excessive reaction to stimulation is expected.

30.4 The Short GnRH Antagonist Protocol

30.4.1 Fixed Dose Protocol

Following the initiation of gonadotropins on day 3 of the menstrual cycle, antagonists, such as cetrorelix/ganirelix, are administered in a fixed dose (0.25–0.5 mg/day) when one or more follicles are ≥14 mm until hCG administration.

30.4.2 Flexible Dose Protocol

Following the initiation of gonadotropins on day 3 of the menstrual cycle, antagonists, cetrorelix/ganirelix, are administered in a flexible protocol (0.25 mg/day) when the leading follicles are 14 mm until hCG administration.

30.4.3 Low-Dose Protocol

Studies testing the effectiveness of GnRH antagonists in controlled ovarian stimulation (COS) for intrauterine insemination (IUI) have provided controversial results. A very recent study was undertaken to evaluate whether the use of a half of the conventional dose of the GnRH antagonist cetrorelix can be effective in increasing the successful rate of IUI cycles [48]. Patients started COS with human menopausal gonadotropin (hMG) on day 3 of the menstrual cycle. Cetrorelix was started when at least one follicle of ≥14 mm was detected in the ultrasound scan, according to the flexible multiple daily

dose protocol, and continued until the trigger day with recombinant hCG. Patients adopting GnRH antagonist at low dose had a pregnancy rate (21.7%) that was significantly higher ($p < 0.05$) in comparison to women receiving hMG only (8.7%). These results suggest that adding a reduced dose of GnRH antagonist to the COS for IUI cycles significantly improves the outcome of the procedure [48].

30.4.4 Scheduling IUI with GnRH Antagonists

In Spanish public hospital reproduction units, it is very problematic to perform programmed intrauterine insemination (IUI) on weekends, if indicated. Small previous pilot studies suggest that using a GnRH antagonist to avoid an LH weekend surge would allow to perform IUI on the following Monday, not impairing the expected pregnancy rate [49]. Between first January 2007 and 31st December 2015, 4.782 intrauterine inseminations were performed at Valladolid University Clinic, Spain, corresponding to 1.650 women. Of them, 911, corresponding to 695 women, should ideally have been performed during the weekend. If it happened that a member of the reproduction unit was on duty during that particular weekend, the standard protocol was not interrupted and the IUI performed as planned (control group, 685 IUIs). If the former was not the case, the weekend gap was bridged by administering 0.25-mg GnRH antagonist (GnRHa). Ovulation was induced by means of 250-μg recombinant HCG (rHCG) 36 h prior to IUI on the following Monday (study group, 226 IUIs). There were no differences in the clinical pregnancy rate (13.7% vs. 16.2%, $p = 0.371$) or in the ongoing pregnancy rate between groups (11.9% vs. 14.9%, $p = 0.271$). The multiple pregnancy rate was also comparable in both groups (14.7% vs. 18.5%, $p = 0.77$). Women with a planned IUI which cannot be performed at the ideal date can be offered postponement for 2 days with the support of GnRHa treatment, with results that are not inferior to those expected applying the regular protocol [49].

30.4.5 The Long GnRH Agonist Protocol

The long agonist protocol involves the subcutaneous (s.c.) administration of 0.1 mg Decapeptyl from the midluteal phase (the 21st day of the cycle preceding the proper stimulation cycle) and until hCG administration. Optimally, gonadotropin stimulation is initiated following pituitary downregulation, identified by estradiol (E2) levels <50 pg/mL and no ovarian follicle larger than 14 mm. However, stimulation may be started if there is a follicle >14 mm with E2 levels <50 pg/mL. Gonadotropins may be administered in a step-up or step-down protocol [32].

30.4.6 Ultra-long Agonist Protocol

The ultra-long agonist protocol (ULP) involves the subcutaneous (s.c.) administration of a single injection of 3.75 mg Decapeptyl followed by daily s.c. Decapeptyl (0.1 mg) administration 4 weeks later and until hCG administration. Gonadotropin stimulation is initiated once pituitary desensitization is achieved as assessed by serum E2 levels ≤100 MIU/mL. hCG, 10,000 IU, is administered when one or more leading follicles are ≥18 mm followed by IUI on evidence of follicular rupture on TVS or 34–36 h after hCG administration. A protocol of recombinant FSH (50 IU daily) and ganirelix (0.25 mg) fixed dose protocol is reported to represent an effective and safe regimen for ovulation induction, associated with a lower rate of mono-ovulation (53.3% vs. 78.8%, $P = 0.06$) and higher CPR per initiated cycle (34.4% vs. 5.9%, $P = 0.005$) compared to alternate-day FSH treatment in couples with unexplained infertility or moderate male subfertility [50]. However, the efficacy of the antagonist in preventing premature luteinization is disputed with some studies reporting in favor [51] and others against [52, 53].

Kutlu et al. assessed the relationship between the estrogen-progesterone alterations before and after ovulation trigger and treatment success in intrauterine insemination (IUI) cycles [54]. Two hundred fifty-one women with infertility underwent ovulation induction followed by IUI. For all subjects, estradiol and progesterone concentrations were evaluated on the trigger and IUI day. The results were analyzed to assess the relationship between hormone levels and positive pregnancy test. There were 34 women with a positive pregnancy test following controlled ovarian stimulation and IUI cycle. Estradiol and progesterone levels on the trigger day and the day of IUI were compared within groups with and without positive pregnancy tests. The comparison revealed significantly increased levels of progesterone after trigger in both groups; however, although there were estradiol level drops in both groups, the drop in the group with negative pregnancy tests was statistically significant. Significant drops in estradiol concentrations after ovulation trigger are associated with IUI cycle treatment failure [54].

30.4.7 Expectant Management

Some randomized trials report that COH-IUI and unstimulated IUI do not offer substantial benefit in couples with unexplained subfertility in terms of CPRs, OPRs, or live birth rates compared with expectant management, and expectant management for a period of 6 months therefore appears justified in these couples [55, 56].

30.5 Semen Parameters and Processing

30.5.1 Semen Parameters to Consider

Semen parameters that must be considered in an IUI program include the semen processing time, processed total motile sperm count, rapid progressive motility after processing, sperm morphology before and after processing, inseminating motile sperm count (IMSC), IUI insemination time, and 24-h sperm survival.

Delaying semen processing from 30 min up to 1 h and/or delaying IUI from 90 min up to 2 h after collection compromises the pregnancy outcome in gonadotropin-IUI cycles [57]. A universal threshold level above which IUI can be performed with acceptable pregnancy rates has not been determined yet [7]. However, IUI success may be impaired in couples with processed total motile sperm (PTMS) count <ten million [58], sperm survival <70% [59], <5% normal spermatozoa, inseminating motile count (IMC) <1 × 106 [7], and prewash IUI-semen pregnancy score (IUI-SPS) <150 [60], necessitating alternative therapy. The PTMS count has been independently associated with fertility after IUI ($P = 0.0014$) [58]. PTMS count ≥10 × 106; their 24-h sperm survival threshold of ≥70% [59]; normal morphology before sperm separation ≥15.5% [odds ratio (OR) = 2.2 ($P = 0.02$)]; rapid, progressive motility ≥25.5% after sperm separation ($P = 0.029$); and curvilinear velocity (VCL) after sperm separation ≥102.65 μm/s ($P = 0.002$) independently predict pregnancy outcome in patients with male factor infertility [61]. These variables would be helpful in counseling patients for future management [61].

There are many studies showing that more days of sexual abstinence increased sperm concentration; however, the direct influence between the days of abstinence and pregnancy rates has not been evaluated. The usual recommendation is 3–4 days prior to intrauterine insemination; this is based on the interval that maximizes the number of motile sperm in the ejaculate. There are some reports with better success rate when abstinence is less than 3 days. Kably-Ambe et al. evaluated the pregnancy rate post-intrauterine insemination according to days of sexual abstinence prior to obtaining semen sample [62]. They published a retrospective, observational, and transversal study in patients attending the Mexican Center for Fertility (CEPAM) for intrauterine insemination. For analysis, patients were grouped by age group, success rate, and days of sexual abstinence. A total of 3123 couples were included, and increased success rate for intrauterine insemination was obtained with less than 7 days of sexual abstinence. The rate of sperm retrieval is inversely proportional to the days of abstinence. A better pregnancy rate in intrauterine insemination was achieved with less than 7 days of sexual abstinence, and sperm retrieval rate was also recorded with fewer days of abstinence [62].

The parameters measured in the standard semen analysis may be insufficient for exact differentiation between fertile and infertile men. Therefore, Boyraz et al. assumed that the high rate of apoptotic sperm in ejaculate may play a role on the etiology of unexplained infertility [63]. Couples with unexplained infertility treated by ovulation induction and intrauterine insemination were consecutively enrolled ($n = 94$). To determine the proapoptotic sperm rate, the ejaculate from patients was stained with annexin V. Thirteen of the 94 couples (13.8%) conceived after intrauterine insemination. The annexin V-positive sperm rate was found to be 20.0% in the whole group. In women failing to conceive, the annexin V-positive sperm rate was 20.8% compared to 15.7% in patients who achieved pregnancy [63]. Although there is a trend toward higher preapoptotic sperm rate in couples failing to get pregnant with insemination, the difference did not reach statistical significance [63].

30.5.2 Semen Preparation Techniques

The rationale behind semen preparation techniques for assisted reproduction is the separation of motile morphologically normal spermatozoa from leucocytes, bacteria, and dead spermatozoa that produce oxygen radicals that negatively influence the ability of sperm to fertilize the egg [64]. There are several sperm separation techniques, and these are based on different principles like migration, filtration, or density gradient centrifugation (DGC). However, the kind of technique employed entirely depends on the quality of the semen ejaculate (i.e., count, motility, morphology, sperm survival, vitality) at an initial evaluation, prior to processing the sample. An ideal preparation technique would be rapid and inexpensive, would isolate all the motile sperms without damaging their fertilizing capacity, and would ensure that the adequate number of motile, functionally normal spermatozoa is present at the site of fertilization following IUI.

The double DGC with different media (IxaPrep, Nycodenz, Sil-Select, PureSperm, or Isolate) and the swim-up technique are the standard techniques in use and have been detailed in other chapters in the book. The swim-up technique (with or without centrifugation) depends entirely on the initial sperm count and percentage of progressive motility. Though swim-up yields a high number of progressively motile sperm and effectively separates sperm from bacteria and cell debris, it is of limited use in cases of low sperm count. DGC involves the use of different density gradients to separate cells by the density during centrifugation. It may be used to obtain a high yield of motile spermatozoa from poor-quality samples with borderline to moderate oligozoospermia/asthenozoospermia. Discretion at this point is very essential. It must be remembered that while a single sperm defect, i.e., a poor count, may be rescued with DGC, a combination of defects will yield poor postprocess results. The glass wool filtration technique may also be used for a high yield of motile spermatozoa. Owing to concerns that centrifugation increases reactive oxygen species (ROS) formation in semen and that high levels of ROS are associated with sperm membrane injury through spontaneous lipid peroxidation, which may alter sperm function, ejaculates with ROS production should not be centrifuged. The time of centrifugation is more important than the g-force for inducing ROS formation in semen, and a shorter centrifugation period in the preparation of sperm for assisted reproductive techniques may be beneficial [28].

Swim-down, refrigeration/heparin techniques, and filtration methods, such as the SpermPrep method and membrane-based electrophoretic filtration system [Cell Sorter-10 (CS-10)], are some of the other techniques that have been in use. The SpermPrep method is a Sephadex preparation with a different bead size that offers quicker, improved semen

manipulation for patients with oligozoospermia and/or asthenozoospermia [65], while the Cell Sorter-10 (CS-10) preferentially isolates spermatozoa with very low levels of DNA damage. Non-centrifugation methods include a novel Zech device that is a dual-chamber capillary dish that obviates the potential harmful effects of centrifugation on sperm.

30.5.3 Advanced Sperm Preparation Methods

The advanced methods for semen preparation include molecular sperm selection strategies such as hyaluronic acid-mediated sperm selection, annexin V magnetic-activated cell separation (MACS) that utilizes colloidal superparamagnetic microbeads (approximately 50 nm in diameter) conjugated with annexin V to separate apoptotic and non-apoptotic spermatozoa, and annexin V molecular glass wool filtration [66].

30.5.4 Density Gradient Centrifugation Versus Swim-Up/Sperm Wash

DGC techniques, utilizing PureSperm, yield significantly higher median total motile sperm counts (TMSC) (32.2 × 106 vs. 17.6 × 106), higher recovery rates of mature motile sperm (69.2% vs. 50.0%), longevity at 4 h (83.0% vs. 55.0%), and postthaw recovery and better preserve semen quality in fresh and cryopreserved semen compared to swim-up [67]. They yield higher pregnancy rates compared to sperm wash (14.3% vs. 11.6%, respectively; 18% vs. 4%, respectively, for samples with <22 million motile sperm in the inseminate) [68] and significant sperm enrichment compared to isolate (50% and 90%) [sperm recovery rates (30.0% vs. 19.7%, respectively)] [67]. Swim-up yields a significantly higher mean percentage of viable sperm but significantly lower recovery rates of total motile, progressively motile, and viable sperm compared with DGC despite similar low rates of apoptotic sperm [69]. Hence, while samples with an acceptable number of motile sperm can be processed efficiently by wash only, poor-quality semen samples should be processed using DGC. The percentage of sperm in the original semen sample with a velocity of ≥80 μm/s for the wash method influences the pregnancy outcome [68]. However, a meta-analysis of five parallel RCTs failed to demonstrate a significant benefit of one technique over the other and concluded insufficient evidence to recommend any specific preparation technique. Further, large high-quality randomized trials are warranted [70].

The combination of DGC and MACS is reported to be superior to all other sperm preparation methods, providing motile, viable, and nonapoptotic spermatozoa [64], improved cryosurvival rates [71], significant improvement in induced acrosome reaction test (IART) ($P < 0.001$) [72] with an improved ability to fertilize eggs using the hamster oocyte penetration assay [66], and significantly reduced levels of apoptotic markers versus DGC alone ($P < 0.001$) [72]. Because of a wide variety of ejaculate qualities, which influence the functions of spermatozoa to a great extent, the efficacy of different preparation methods will vary; hence, the technique to be used must be individually tailored.

30.6 IUI in Practice: Description of the Procedure

30.6.1 Timing of Insemination

IUI may be timed 34–36 h after the administration of 10,000 IU hCG intramuscularly, which may be timed following evidence of one or more follicles ≥18 mm during ultrasound monitoring of folliculogenesis and an endometrial thickness >7 mm (with triple-line development). Alternatively, IUI may be timed following sonographic evidence of follicular rupture and free fluid in the pouch of Douglas, or following biochemical evidence of an LH surge. A meta-analysis of seven controlled trials (2623 patients), examining the effectiveness of hCG administration before IUI on CPRs in comparison with LH detection, failed to demonstrate a consistent, clinically important benefit of hCG-induced ovulation compared with LH monitoring of spontaneous ovulation for IUI timing [73]. Despite comparable pregnancy rates between hCG-induced ovulation and LH surge-timed IUI (an increase in LH level ≥ 200% over a mean of preceding 2 days), a significantly longer time to IUI, significantly higher cancelation rates (31% vs. 11%) attributed mainly to failure to detect an LH surge [74], and favorable outcomes limited only to CC-induced cycles [75] have been reported, suggesting that the decision to use hCG for IUI timing should be influenced by factors other than pregnancy rates [74]. Postponing IUI until the observation of follicle rupture at transvaginal ultrasonography may yield a higher pregnancy rate (23.5% vs. 8.8%, respectively; $p < 0.001$) in couples with unexplained infertility and male factor subfertility [76].

With regard to the ovulation-inducing agent, a subcutaneous injection of a relatively low dose of GnRHa [triptorelin (Decapeptyl), 0.1 mg] can be as effective as hCG (10,000 IU) after follicular maturation in producing pregnancy in COH-IUI treatment cycles in patients presenting with amenorrhea, oligomenorrhea, or unexplained infertility with comparative conception rates (15.3% vs. 26.5%), abortion rates (18.2% vs. 33.3%), and term pregnancy rates (13.6% vs. 19.0%), respectively [77].

Giugliano et al.'s aim was to assess the impact of the ultrasonographic detection of follicular rupture on the intrauterine insemination success [78]. A total of 313 women undergoing ovarian stimulation for intrauterine insemination were enrolled. Transvaginal ultrasonography was performed to check whether the dominant follicle had ruptured, and according to that the patients were divided into two groups. The ultrasound detection of follicular rupture was observed in 156 patients (54%). The independent variables favoring follicular rupture were age (t: 7.646, $p < 0.0005$),

FSH value (t: −5.637, $p < 0.0005$), duration of infertility (t: −4.265, $p < 0.0005$), and menstrual cycle length (t: −4.927, $p < 0.0005$). Moreover, the logistic regression analysis demonstrated that the predictive variables for follicular rupture were FSH value (OR 1.7, CI 95% 1.3–2.3, $p < 0.0005$), duration of infertility (OR 2.6, CI 95% 1.6–4.2, $p < 0.0005$), and menstrual cycle length (OR 2.4, CI 95% 1.7–3.4, $p < 0.0005$). Pregnancy occurred in 23 patients of the group A (14.7%) and in 22 patients of the group B (16.5%) without a significant difference ($p = 0.6$). The logistic regression analysis confirmed that neither the evidence of follicular rupture nor any other variables influenced the pregnancy rate [78].

Liang et al. investigated the influence of the time interval from the end of semen processing to artificial intrauterine insemination with husband's sperm (AIH-IUI) on the rate of clinical pregnancy [79]. This study involved 191 AIH-IUI cycles with the same ovulation induction protocol. After Percoll density gradient centrifugation, they divided the sperm into four groups based on the incubation time: 0–19, 20–39, 40–59, and 60–80 min and again into another four groups according to the total progressively motile sperm count (TPMC): (0–9), (10–20), (21–30), and > 30 × 10(6). They analyzed the correlation of the clinical pregnancy rate with the time interval from the end of sperm processing to AIH-IUI and with other influencing factors, such as maternal age, infertility duration, and semen quality. The rate of clinical pregnancy was significantly higher in the 20–39-min group (18.3%) than in the 0–19-, 40–59-, and 60–80-min groups (12.7%, 11.4%, and 9.1%) (all $P < 0.05$). The (10–20) × 10(6) group achieved a remarkably higher pregnancy rate (16.7%) than the (0–9), (21–30), and > 30 × 10(6) groups (0%, 11.4%, and 8.3%) (all $P < 0.05$). Logistic multivariate analysis showed that the rate of clinical pregnancy was decreased with the increased age of women (OR 0.89, 95% CI 0.83–0.94) but significantly elevated in the 20–39-min group (OR 2.11, 95% CI 1.34–3.13) and in (10–20) × 10(6) group (OR 2.06, 95% CI 1.32–3.46). The time interval from the end of sperm processing to AIH-IUI is a significant factor influencing the rate of clinical pregnancy of AIH-IUI [79].

Çok et al. reported on the comparison of the effect of preserving prepared sperm samples at room temperature or at 37 °C before intrauterine insemination (IUI) on clinical pregnancy rate [80]. Clinical pregnancy rates were similar in IUI cycles in which prepared sperm samples were preserved at 37 °C and at room temperature (9.3% vs. 8.9%). Clinical pregnancy rates in IUI cycles with two follicles were higher than IUI cycles with one follicle (10.8% vs. 7.6%) ($p = 0.002$). Further statistical analysis after splitting data according to the number of the follicles revealed that there was no statistical difference between clinical pregnancy rates after IUI cycles in which the prepared sperm samples were preserved at 37 °C or at room temperature in both one-follicle (7.6% vs. 7.6%) and two-follicle cycles (11.5% vs. 10.1%). Preserving prepared sperm samples at room temperature had no negative effect on clinical pregnancy rates when compared with preserving prepared sperm samples at 37 °C during IUI cycles [80].

30.6.2 Insemination Procedure

IUI is performed with the patient in the dorsal lithotomy position on an empty bladder. The Cusco speculum is inserted, the cervical os is identified, and the cervix is cleaned with a swab of sterile saline. The os may be held in place with a vulsellum, if necessary. Meanwhile, in the laboratory, an atraumatic Wallace ET catheter is loaded with 0.5 mL of processed sperm. The catheter is inserted into the uterine cavity without touching the fundus, and the sperm suspension is gradually released. The patient is advised to rest in the same position for 15–20 min prior to discharge. Immobilization for 15 min after insemination yields significantly higher OPR per couple (27% vs. 18%) and live birth rates (27% vs. 17%) compared to the control group [81].

30.6.3 Ultrasound-Guided IUI Versus Blind IUI

Polat et al. performed a study to determine the effects of ultrasound (US) guidance during intrauterine insemination (IUI) on pregnancy rate [82]. The prospective study enrolled 130 couples who were scheduled to undergo IUI. The couples were randomized according to a computer-generated list into two groups: (1) the ultrasound-guided IUI group included 64 couples ($n = 64$) treated for 99 cycles and (2) the blind IUI group included 66 couples ($n = 66$) treated for 104 cycles. All women underwent controlled ovarian stimulation before IUI. The study's main measurements were pregnancy rate per cycle and pregnancy rate per woman. The pregnancy rates were similar in both the ultrasound-guided (USG) (16.2%, 16/99) and non-ultrasound-guided (NUSG) (12.5%, 13/104) groups ($p = 0.386$). The present results suggest a routine ultrasound guidance during IUI is not essential as it does not increase pregnancy rates but it can be used in such cases to overwhelm some sort of difficulties [82].

30.6.4 Luteal Phase Support

Following IUI, luteal phase support may be achieved with transvaginal administration of micronized progesterone (200 mg/day) or vaginal progesterone gel (Crinone 8% gel) for 2 weeks. A clinical pregnancy may be documented following sonographic evidence of a gestational sac with heartbeat at 6 weeks of amenorrhea and an ongoing pregnancy by >12 weeks of amenorrhea. Luteal phase support with vaginal progesterone gel (Crinone 8% gel) significantly affects the success of ovarian stimulation and IUI cycles in patients with unexplained infertility, yielding significantly higher CPR/cycle and CPR/patient (21.1% and 39.4%, respectively) compared to patients who received no luteal phase support (12.7% and 23.8%, respectively) [83]. Alternatively, intranasal administration of a GnRH agonist (buserelin) could be effective in providing luteal support with good pregnancy rates (28%) [84].

30.7 Post-procedure Care

Patients can pursue their routine activity following IUI but must take care to avoid excess physical exertion. Signs of spotting or bleeding must be reported immediately.

30.7.1 Single Versus Double Insemination

Studies have reported significantly better pregnancy outcomes following double insemination (12 and 34 h after hCG administration) versus single insemination (34–36 h after hCG administration) in couples with ovulatory dysfunction, endometriosis, male factor, unexplained infertility, tubal factor infertility, and combined diagnoses, particularly within gonadotropin protocols and ovulatory dysfunction and male factor diagnostic categories, and despite a significantly lower female age in the single IUI group [85] and slightly higher cost of double IUI [86]. However, a recent meta-analysis of six RCTs, involving 829 women, failed to demonstrate a clear benefit of double IUI versus single IUI in the overall CPR in couples with unexplained infertility [87]. Despite the 36th h being the preferred timing for IUI, no statistical difference regarding pregnancy rates has been reported between single 24th-h and double 12th- and 36th-h inseminations in patients with unexplained infertility, male factor, and ovulatory dysfunction following COH with gonadotropin + IUI, suggesting that the 24th-h IUI might be preferred in demanding situations [88].

30.7.2 IUI Versus Timed Intercourse (TI)

Despite evidence of a significant benefit of IUI over TI in two large meta-analyses in terms of the probability of conception, both in natural cycles and in COH cycles, with COH (gonadotropins)-IUI offering the best chance of conception in couples with male subfertility [29] and ovulatory infertility [89], a recent meta-analysis [90] failed to demonstrate a significant clinical benefit in terms of pregnancy rates between stimulated and natural cycle IUI, natural cycle TI/IUI, and stimulated IUI/TI. It concluded insufficient data to perform a statistical analysis or to recommend or advise against IUI with or without COH above TI, or vice versa, or to document adverse outcomes such as OHSS, multiple pregnancy, miscarriage rate, and ectopic pregnancy [90]. Timed intercourse following IUI with a low number of motile sperm is an alternative treatment that appears to be a practical, simple, and an inexpensive addition that significantly increases the pregnancy rates over IUI alone (27.7% vs. 10.5%, $P = 0.023$) in infertile couples with a normal sperminogram [91].

30.7.3 Number of Cycles

There is general agreement in the literature that four to six IUI cycles may be performed with acceptable pregnancy rates before resorting to alternative therapy [68, 92] though studies have reported acceptable OPR in high-order IUI cycles up to nine [27]. More than four cycles of CC-IUI can compensate for low pregnancy rates due to age, semen quality, or follicle number in patients with ovulation dysfunction. Significantly lower mean PRs have been reported in patients ≥43 years with poor semen quality, single preovulatory follicles, and diagnoses other than ovulatory dysfunction compared to those after four (CPRs 46% for ovulatory dysfunction; 38% for cervical factor, male factor, and unexplained infertility; 34% for endometriosis; and 26% for tubal factor infertility) or six (CPRs 65% for ovulation dysfunction, 35% for endometriosis, and unchanged for other diagnoses) cycles [92]. Morshedi et al. [68] reported 88% of pregnancies in the first three cycles of IUI and 95.5% within the first four cycles with an overall CPR/cycle of 13.0% and CPR/patient of 28.3% with a miscarriage rate of 34.0% [68]. Aboulghar et al. [93] reported significantly higher cycle fecundity in the first three trials of COH-IUI than in cycles four to six (16.4% vs. 5.6%, $P < 0.001$, $n = 594$, 1112 cycles), suggesting that COH-IUI for the treatment of unexplained infertility should be limited to a maximum of three trials and patients should be offered IVF or ICSI if they fail to conceive after three trials of COH and IUI [93]. Moreover, Qublan et al. [94] reported an increased incidence and recurrence rate of luteinized unruptured follicle (LUF) among patients with pre-existing LUF undergoing multiple IUI cycles. The incidence of LUF was 56.5% in the second cycle of IUI treatment with a recurrence rate of 78.6% and 58.9% among patients who underwent three consecutive IUI cycles with a recurrence rate of 90%. In these patients, other options of infertility treatment might be justified [94]. The use of clomiphene citrate for unexplained fertility for more than 12 cycles of IUI has been associated with a threefold increase in risk of ovarian cancer [95].

30.7.4 Intentional Endometrial Injury and Intrauterine Insemination

Intentional endometrial injury is currently being proposed as a technique to improve the probability of pregnancy in women undergoing assisted reproductive technologies (ARTs) such as in vitro fertilization (IVF). Endometrial injury is often performed by pipelle biopsy or a similar technique and is a common, simple, gynecological procedure that has an established safety profile. However, it is also known to be associated with a moderate degree of discomfort/pain and requires an additional pelvic examination. The effectiveness of this procedure outside of ART, in women or couples attempting to conceive via sexual intercourse or with low complexity fertility treatments such as intrauterine insemination (IUI) and ovulation induction (OI), remains unclear.

Lensen et al. set up a study to evaluate the effectiveness and safety of intentional endometrial injury in subfertile women and couples attempting to conceive through sexual intercourse or intrauterine insemination (IUI) [96]. They searched the Cochrane Gynaecology and Fertility Group

Specialised Register, Cochrane Central Register of Controlled Trials (CENTRAL), MEDLINE, Embase, PsycINFO, CINAHL, LILACS, DARE, ISI Web of Knowledge, and ► ClinicalTrials.gov, as well as reference lists of relevant reviews and included studies. They performed the searches from inception to 31 October 2015. They included randomized controlled trials (RCTs) that evaluated any kind of intentional endometrial injury in women planning to undergo IUI or attempting to conceive spontaneously (with or without OI) compared to no intervention, a mock intervention, or intentional endometrial injury performed at a different time or to a higher/lower degree. Two review authors independently selected trials, extracted data, and assessed trial quality using GRADE methodology. The primary outcomes were live birth/ongoing pregnancy and pain experienced during the procedure. Secondary outcomes were clinical pregnancy, miscarriage, ectopic pregnancy, multiple pregnancy, and bleeding secondary to the procedure. We combined data to calculate pooled risk ratios (RRs) and 95% confidence intervals (CIs). Statistical heterogeneity was assessed using the I^2 statistic. Nine trials, which included a total of 1512 women, met the inclusion criteria of this Cochrane review. Most of these studies included women with unexplained infertility. In seven studies, the women were undergoing IUI and in two studies, the women were trying to conceive through sexual intercourse. Eight trials compared intentional endometrial injury with no injury/placebo procedure; of these, two trials also compared intentional endometrial injury in the cycle prior to IUI with intentional endometrial injury in the IUI cycle. One trial compared higher vs. lower degree of intentional endometrial injury. The study is uncertain whether endometrial injury improves live birth/ongoing pregnancy as the quality of the evidence has been assessed as very low (risk ratio (RR) 2.22, 95% confidence interval (CI) 1.56 to 3.15; six RCTs, 950 participants; I^2 statistic = 0%, very low quality evidence). When they restricted the analysis to only studies at low risk of bias, the effect was imprecise and the evidence remained of very low quality (RR 2.64, 95% CI 1.03 to 6.82; one RCT, 105 participants; very low quality evidence). Endometrial injury may improve clinical pregnancy rates; however, the evidence is of low quality (RR 1.98, 95% CI 1.51 to 2.58; eight RCTs, 1180 participants; I^2 statistic = 0%, low quality evidence). The average pain experienced by participants undergoing endometrial injury was 6/10 on a 0–10 visual analogue scale (VAS) (standard deviation = 1.5). However, only one study reported this outcome. When the analysis compared hysteroscopy with endometrial injury to hysteroscopy alone, there was no evidence of a difference in ongoing pregnancy rate (RR 1.29, 95% CI 0.71 to 2.35; one RCT, 332 participants; low quality evidence) or clinical pregnancy rate (RR 1.15, 95% CI 0.66 to 2.01; one RCT, 332 participants, low quality evidence). This study did not report the primary outcome of pain during the procedure. When endometrial injury was performed in the cycle prior to IUI compared to the same cycle as the IUI, there was no evidence of a difference in ongoing pregnancy rate (RR 0.65, 95% CI 0.37 to 1.16; one RCT, 176 participants; very low quality evidence) or clinical pregnancy rate (RR 0.82, 95% CI 0.50 to 1.36; two RCTs, 276 participants; very low quality evidence). Neither of these studies reported the primary outcome of pain during the procedure. In all three comparisons, there was no evidence of an effect on miscarriage, ectopic pregnancy, or multiple pregnancy. No studies reported bleeding secondary to the procedure. It is uncertain whether endometrial injury improves the probability of pregnancy and live birth/ongoing pregnancy in women undergoing IUI or attempting to conceive via sexual intercourse [96]. Further well-conducted RCTs that recruit large numbers of participants and minimize internal bias are required to confirm or refute these findings.

30.8 Intrauterine Versus Other Forms of Artificial Insemination

30.8.1 Intracervical Insemination

Sperm may be deposited in or around the endocervical canal (cervical insemination—CI) or in the uterine cavity itself (IUI). IUI has been considered potentially more effective than CI as the sperm bypass the cervical mucus and are deposited closer to the fallopian tubes. The cost and risks of IUI, on the other hand, may be higher because of the need for sperm preparation, the introduction of foreign material into the uterus [97], and the consequent risk of infection and anaphylaxis [98]. Significantly improved pregnancy rates and live birth rates without a statistically significant evidence of an effect on multiple pregnancies or miscarriages have been reported following six cycles of stimulated IUI with CC/gonadotropins using cryopreserved sperm in comparison to cervical insemination [97].

30.8.2 Slow Release Intrauterine Insemination

A modified application technique of intrauterine insemination (IUI) is slow release insemination (SRI), first described by Muharib et al. [99], who postulated higher pregnancy rates with a slow release of spermatozoa for 3 h. To investigate this approach, two randomized controlled, crossover pilot studies were performed from 2004 to 2006 in Israel and Germany to compare SRI with the standard bolus IUI. Marschalek et al. aimed to present the results and perform a meta-analysis on available data for SRI [100]. Univariate comparisons of pregnancy rates were performed using one-tailed Z-tests for method superiority. For meta-analysis, a fixed-effect Mantel-Haenszel weighted average of relative risk was performed. Fifty treatment cycles (IUI, $n = 25$; SRI, $n = 25$) were performed in Germany, achieving four pregnancies (IUI, 4%; SRI, 12%; $p > 0.05$). Thirty-nine treatment cycles (IUI, $n = 19$; SRI, $n = 20$) were performed in Israel, achieving six pregnancies (IUI, 10.5%; SRI, 20%;

$p > 0.05$). Meta-analysis of all eligible studies for SRI ($n = 3$) revealed a combined relative risk for pregnancy after SRI of 2.64 (95% CI 1.04–6.74; $p = 0.02$). In conclusion, these results lend support to the hypothesis that the pregnancy rate might be improved by SRI compared to the standard bolus technique [100].

30.8.3 Fallopian Tube Sperm Perfusion

Fallopian tube sperm perfusion (FSP) is based on the pressure injection of 4 mL of sperm suspension with the attempt of sealing of the cervix to prevent semen reflux [34] and ensures the presence of higher sperm densities in the fallopian tubes at the time of ovulation than does standard IUI [101]. The IUI technique, on the other hand, is based on intrauterine injection of 0.5 mL of sperm suspension without flushing the tubes [30]. Despite previous reports of the advantages of FSP in terms of higher CPR and OPR/patient and reduced cost and complications in couples with unexplained infertility compared to IUI [102, 103], a recent meta-analysis of eight randomized controlled studies involving 595 couples with nontubal subfertility including a subgroup analysis in couples with unexplained subfertility reported no clear benefit for FSP over IUI [101]. In couples with long-standing infertility, intraperitoneal insemination (IPI), FSP, and IUI have similar efficacy in the achievement of a clinical pregnancy [104].

Intrauterine tuboperitoneal insemination (IUTPI) with 10 mL of inseminate has been proposed as a useful technique in the treatment of unexplained infertility, mild or moderate male infertility, and mild or moderate endometriosis compared to FSP (CPR/cycle 29.4% vs. 17.6%), and three attempts of IUTPI may be beneficial before moving on to more invasive and expensive methods of assisted reproduction [105].

30.9 Risks and Complications of IUI

30.9.1 Early Complications: Risks of the Procedure

Though complications after IUI are rare, studies have reported slight cervical contact bleeding and mild abdominal discomfort and/or cramps [106]. Vaginal administration of misoprostol at the time of IUI is associated with a significant increase in vaginal bleeding and abdominal cramping rates and does not seem to enhance the outcome [107]. Spontaneous abortions, blighted ovum, and ectopic pregnancies have been reported in 7.8%, 2.6%, and 1.3% of the pregnancies, respectively, in COH-IUI cycles [108]. Infectious complications associated with IUI are frequently cited, though rarely reported. According to Sacks and Simon [109], most reported cases of infection fail to show evidence for the actual presence of infection, and the prevalence is unaltered by the administration of prophylactic antibiotics or washing the semen sample with antibiotics. *Escherichia coli* septicemia has been reported subsequent to IUI [110]. *Chlamydia trachomatis* infection, acquired as an intrauterine infection, as well as during transit through the birth channel, may result in a number of adverse pregnancy outcomes, including ectopic pregnancy, early and late abortion, intrauterine infections of the fetus, stillbirth, prematurity, premature rupture of the membranes (PROM), and postpartum endometritis [110].

30.9.2 Late Complications

Although IUI itself is less invasive and expensive than other techniques of assisted reproduction, the adverse effects of COH, such as OHSS and multiple pregnancies, are a concern [22, 24, 90]. Miscarriage rates ranging from 11.8% [6] to 34.0% [4] have been reported following COH-IUI.

30.9.3 Multiple Births

Multiple pregnancy rates following IUI are significantly influenced by age and etiology of the patient. Multiple pregnancy rates of 6.0% in unexplained and male subfertility [33], 13.2% in anovulatory infertility [111], and 23.5% in couples with exclusive female factors, such as anovulation, cervical factors, or unexplained infertility and a maximum of three IUI cycles, have been reported [112]. Multifollicular growth is associated with increased pregnancy rates following COH-IUI. Surpassing the recruitment of two follicles would lead to a dramatic increase in the risk of OHSS and multiple pregnancies without a substantial gain in overall pregnancy rate [113, 114]. According to van Rumste et al. [113], one stimulated follicle should be the goal if safety is the primary concern, whereas two follicles may be accepted after careful patient counseling. They reported an absolute pregnancy rate of 8.4% for monofollicular growth and 15% for multifollicular growth. Compared with monofollicular growth, pregnancy rates increased by 5% and 8%, while the risk of multiple pregnancies increased by 6%, 14%, and 10% when stimulating two, three, and four follicles. The absolute rate of multiple pregnancies was 0.3% after monofollicular growth and 2.8% after multifollicular growth [113]. In patients <35 years old, the incidence of multiple pregnancies, particularly ≥3 implantations, tripled when ≥6 follicles were ≥ 12 mm in CC, hMG, and CC + hMG cycles and when E2 levels were > 1000 pg mL in hMG and CC + hMG cycles. In patients ≥35 years, pregnancy rates in hMG and CC + hMG cycles doubled when six or more follicles were ≥ 12 mm or E2 levels were > 1000 pg mL, whereas three or more implantations were not significantly increased [114].

Treatment strategies to reduce the incidence of multiple pregnancies in ovulation induction programs can be targeted to reduce multiple follicular development and subsequent ovulation by a more aggressive cancelation policy, follicle reduction by fine needle aspiration, conversion to IVF, or

dealing with the problem of multiple gestation after it has occurred (i.e., multifetal pregnancy reduction) [115]. Specifically, tailoring the rate of multifollicular development according to the duration and type of infertility (etiology, primary or secondary, female age) would prove to be a safer approach for achieving pregnancy as well as avoiding adverse effects such as the risk of OHSS and multiple pregnancies [116]. Withholding hCG or IUI in CC, hMG, and CC + hMG cycles when six or more follicles are ≥12 mm may reduce triplet and higher-order implantations by 67% without significantly reducing pregnancy rates for patients under 35 years of age [101]. According to Ragni et al. [117], in COH-IUI cycles, daily administration of 50 IU recombinant FSH with the use of GnRH antagonists and a policy of strict cancelation (if three or more follicles ≥16 mm and/or five or more follicles ≥11 mm) based on echographic criteria are associated with a satisfactory pregnancy rate per initiated cycle and a low risk of high-order multiple pregnancies. The CPR per initiated cycle was 9.2% with a 9.5% and 0% incidence of twins and high-order multiple pregnancies, respectively [117].

30.10 Success Rates with IUI

30.10.1 Factors Affecting Outcome

Overall CPRs/cycle ranging from 9.2% to 22% have been reported in the literature following stimulated IUI [4, 6, 7, 89, 109] with live birth rates of 10% for CC-stimulated and 8.7% for rFSH-stimulated cycles [37]. Guven et al. [118] reported a PR of 7.9% in the primary infertility group and 21.4% in the secondary infertility group. However, these results are largely determined by the patient selection criteria, the sperm and follicular characteristics, and the stimulation protocol in the various studies.

Characterization of prognostic factors for pregnancy is essential, particularly those for women at risk of multiple pregnancies after IUI [119].

30.10.2 Favorable Predictors of IUI Outcome

The following factors have been reported as significant predictors of the pregnancy outcome following IUI:

- Patient's age ≤ 35 years, irrespective of the method of sperm preparation used [68]. A higher birth rate (25.8%) has been reported in women <35 years undergoing COH-IUI compared to women ≥35 years (14.0%) when four or more follicles were greater than or equal to 12 mm [120].
- Infertility etiology. Pregnancy rates/cycle may vary among women with different etiologies, being highest in anovulatory infertility (19.2%) and lowest in endometriosis-based infertility (11.9%). The cumulative pregnancy rates varied greatly by diagnosis from 16% for patients with male factor infertility to 60% for patients with ovulatory disorder [121]. Cervical factor yields a favorable outcome [122].
- Sperm parameters.

Total motile sperm count (TMSC) before sperm preparation ($P > 0.05$) [118].

Sperm motility. Patients with original sperm motility ≥30% had a higher cumulative pregnancy rate (74%) than patients with motility <30% ($P < 0.005$) with a four-times increase in PR with an increase in motility of ≥30% [123].

Progressive motility [112].

Normal sperm morphology [118, 119]. Significantly higher PRs have been reported with samples with normal sperm morphology of >4% (according to Kruger's criteria) compared to ≤4% (22.2% vs. 6.7%, respectively; $p = 0.003$) [118].

Processed total motile sperm (PTMS) count ≥10 × 106 and 24-h survival of ≥70% can predict the IUI outcome with 94% sensitivity and 86% specificity [2]. An average TMSC of ten million may be a useful threshold value for decisions about treating a couple with IUI or IVF [124].

- Mild controlled ovarian hyperstimulation [120, 122, 125]. A significantly higher incidence of follicles (81% vs. 53%; $P < 0.05$) as well as cycles (63% vs. 49%; $P < 0.05$) with uniformly high-grade vascularity has been observed by transvaginal power Doppler ultrasonography on the day of insemination (32–36 h post-hCG administration) in COH-IUI cycles compared with unstimulated or clomiphene-induced donor cycles [125].
- Follicular dynamics.

Increase in the number of preovulatory follicles (1–4) at the time of hCG administration [2, 112, 122, 126].

Increase in AFC and, hence, increase in the dominant follicle number and E2 level on the day of hCG administration [7].

Serum E2 levels. Birth rate increased from 3.6% when E2 was <500 pg/mL to 19.6% when E2 was ≥2500 pg/mL [122].

High-grade follicular vascularity. Higher pregnancy and lower early pregnancy loss rates have been observed in cycles with uniformly high-grade follicular vascularity, possibly attributed to COH, compared with other vascularity grades [125]. However, a recent study by Ragni et al. [127] reported that follicular vascularity (follicles with a mean diameter ≥ 16 mm) does not predict the chance of pregnancy in women undergoing mild COH and IUI [127].

- Endometrial thickness [112].
- Shorter duration of infertility [112].
- Cervical mucus aspiration before IUI [66].

The etiology of infertility and the stimulation protocol used may have a significant effect on the pregnancy outcome. Pregnancy rates of 36%, 25.0%, and 30% have been reported following stimulation with sequential CC and hMG and three follicles in patients with anovulatory infertility, male infertility, and combined infertility, respectively, 21.1% fol-

lowing CC stimulation in endometriosis-based infertility, and 24.3% with an IMC >30 × 106. Ovarian stimulation in unexplained infertility resulted in PRs of 24.2% and 19.8% with three follicles and an IMC >30 × 106, respectively [111].

30.10.3 Factors Predicting a Poor Prognosis

A significant negative correlation of the following factors with the IUI pregnancy outcome has been reported:

- Increasing maternal age ≥ 35 [120–122]. A 0.0% cumulative probability of ongoing pregnancy following three cycles of IUI in women ≥40 years has been reported compared to women <40 years (28.2%) irrespective of the infertility history, use of ovarian stimulation, or baseline semen parameters, suggesting that the treatment of male and/or cervical factor by IUI is ineffective for women ≥40 years [128]. Treatment with CC is ineffective, and delivery rates following COH with gonadotropins and IUI are <5%. Therefore, it is recommended that after a short trial of gonadotropins and IUI, women aged 40–41 years should be quickly referred to IVF. At an older age, IVF is the primary treatment option [129].
- Decreased basal AFC. In women >35 years with AFCs <5, the application of COH-IUI may not be indicated [21]. Low basal AFC is associated with lower clinical pregnancy and live birth rates in unexplained subfertile couples treated with COH-IUI [130].
- Male factor infertility with initial sperm motility <30% ($P < 0.002$), initial sperm count <5 × 106 per mL ($P < 0.01$) [127], and normal sperm morphology <14% [125].
- Postwash TMSC ≤20 million/mL [121].
- Endometriosis and tubal factor-associated infertility [119–122].
- Uterine anomalies [122].
- Increasing number of cycles with more than four treatment cycles [120, 122].
- Longer duration of subfertility [122].
- Low midluteal progesterone (P4) level ≤ 25 nmol/L [131].

A recent study by Merviel et al. [132] concluded that the couple with the best chance of pregnancy following COH (gonadotropin)-IUI can be described as a woman <30 years with cervical or anovulatory infertility and a man with a TMS ≥five million spermatozoa. The "ideal" stimulation cycle enables the recruitment of two follicles measuring >16 mm with an E2 concentration > 500 pg/mL on the day of hCG administration. The best results are obtained when IUI is performed using a soft catheter [132]. Though male and female factors contribute to pregnancy outcome, the clinician can influence prognosis by increasing the number of follicles, especially in severe male factor cases [119].

30.10.4 Intrauterine Insemination and Major Congenital Malformations

Multiple pregnancies are a recognized adverse effect of assisted reproductive technologies; nevertheless, there is no consensus on the incremental risk associated with the ovarian stimulation (OS) used alone and intrauterine insemination (IUI). The relationship between OS and IUI and the risk of major congenital malformations (MCM) is unclear. Chaabane et al. set up a study [133] to summarize the literature and evaluate the risk of multiple pregnancy and MCM associated with OS used alone and IUI used with or without OS compared to natural conception (spontaneously conceived infants without any type of fertility treatments). They carried out a systematic review to identify published papers between 1966 and 2014 in MEDLINE, Embase, and the Cochrane Central Register of Controlled Trials. They included observational studies and randomized clinical trials related to the risk of multiple pregnancies and MCM conceived following OS alone or IUI compared to natural conception (spontaneously conceived infants without any fertility treatments). There were 63 studies included in this review. The systematic review suggests that the use of any OS alone was associated with an increased risk of multiple pregnancy compared to natural conception (pooled RR 8.80, 95% CI 5.09–15.20; $p = 0.000$; 9 studies). Similar increases in the risk of multiple pregnancies were observed following clomiphene citrate used without assisted reproductive technologies. Compared to natural conception, the use of IUI with or without OS was associated with an increased risk of multiple pregnancy (pooled RR 9.73, 95% CI 7.52–12.60; p = 0.000; 6 studies). Compared to natural conception, the use of any OS alone was associated with an increased risk of any MCM (pooled RR 1.18, 95% CI 1.03–1.36; 11 studies), major musculoskeletal malformations (pooled RR 1.48, 95% CI 1.21–1.81; 7 studies), and malformations of the nervous system (pooled RR 1.73, 95% CI 1.15–2.61; 6 studies). Compared to natural conception, the use of IUI was associated with an increased risk of any MCM (pooled RR 1.23, 95% CI 1.10–1.37; 10 studies), major urogenital malformations (pooled RR 1.52, 95% CI 1.04–2.22; 7 studies), and musculoskeletal malformations (pooled RR 1.54, 95% CI 1.20–1.98; 7 studies). The increased risk of multiple pregnancy and certain types of MCM associated with the use of less invasive fertility treatments, such as OS and IUI, found in this review, highlights the importance of the practice framing [133].

Heterogeneity in OS protocols, the combination with other fertility agents, the limited number of studies, and the methodological quality differences reduce our ability to draw conclusions on specific treatment. More observational studies, assessing the risk of multiple pregnancy or MCM, as a primary outcome, using standardized methodologies, in larger and better clinically defined populations are needed.

30.10.5 Interesting Case Reports

Bakas et al. [134] reported a rare case of maternal hyperthyroidism after intrauterine insemination due to hypertrophic action of hCG. A 36-year-old woman after successful intrauterine insemination and triplet pregnancy developed hyperthyroidism with resistance to medical treatment. All signs of hyperthyroidism resolved and the results of thyroid function tests returned to normal without any medication after embryo meiosis. De novo maternal hyperthyroidism may develop during pregnancy as a result of pathological stimulation of the thyroid gland from the high levels of hCG hormone that can be seen in multiple pregnancies. The risk of hyperthyroidism is related to the number of fetuses. Reversibility of symptomatology can be seen after fetal reduction of multiple pregnancies [134].

The management of pregnancy in young women with essential thrombocythemia is complex and may present a difficult problem. An adverse pregnancy outcome due to thrombosis or bleeding is a common complication. In addition, little is known about fertility in these women prior to the disease. Leković et al. published the first case of a young woman with primary infertility and essential thrombocythemia who had uneventfully delivered a healthy boy in the 40th week of pregnancy [135]. Her platelet count was normalized during treatment with interferon-alfa. The patient failed to become pregnant in the natural way and after three attempts of programmed intercourse. She conceived only following intrauterine insemination. During pregnancy, the patient was carefully monitored by a hematologist and gynecologist. Intrauterine insemination with minimal hormonal stimulation due to the risk of thrombosis could be recommended as the safest treatment option of infertility in women with essential thrombocythemia [135].

30.10.6 Recent Advances

The transcriptome of spermatozoa used in homologous IUI reveals profound differences between expression profiles of sperm samples that impregnate successfully and those that do not. These differences might improve the predictive power of sperm evaluation to estimate IUI success by complementing the basic sperm analysis [136]. Three-dimensional (3D) and 3D power Doppler (PD) when used with 2D ultrasound and color Doppler for pre-hCG follicular assessment improve pregnancy rates in IUI cycles by enabling an assessment of the follicular volume, perifollicular resistance index, and perifollicular vascularity index, all of which may influence the conception rates [137].

Swierkowski-Blanchard conducted an observational pilot study to determine the impact of the frequency and intensity of uterine contractions (UCs) at the time of IUI on subsequent fertility [138]. One hundred volunteer women scheduled for IUI between April 2011 and July 2013, in whom UCs were assessed during the ultrasound before IUI were included. A two-dimensional sagittal uterus elastography was recorded for 5 min. The elasticity index, defined as the mean ratio of elastographic measurements between the subendometrial area (of interest) and the endometrial area (control), was computed. UC frequency, endometrial thickness and volume, and subendometrial vascularization were also measured. These parameters, along with characteristics of the IUI cycle, were entered into a logistic regression model for predicting ongoing pregnancy. The elasticity index was significantly higher (2.4 ± 1.3 vs. 1.5 ± 0.7, i.e., with stiffer myometrium), and the endometrium was significantly less echogenic in future pregnant women. Factors closely reaching significance were age, previous fertility, day 3 hormonal assessments, number of inseminated spermatozoa, endometrial thickness, and UC count. In multivariate analysis, low UC frequency (<2.8/min; odds ratio [OR] = 0.039), high elasticity index (>1.7; OR = 63.26), high endometrial thickness on the ovulation triggering day (>8 mm; OR = 28.21), and low patient age (<32 years; OR = 0.001) were predictive of pregnancy after IUI. A low frequency and high intensity of UCs at the day of IUI appears associated with a higher pregnancy rate. Elastography provides a promising innovative tool for IUI monitoring [138].

Uterine contractibility is considered a powerful prognostic factor in predicting the embryo transfer outcome. Moreover, uterine contractions are known to be stimulated by prostaglandins which are produced by cyclooxygenase from arachidonic acid. As such, suppressing the inflammatory response and contractions using anti-inflammatory and relaxant agents is expected to result in increased success rate of embryo transfer and artificial insemination. Zarei et al. investigated the effect of piroxicam administration on the success rate in intrauterine insemination (IU) cycles in patients presenting with unexplained infertility [139]. This randomized, placebo-controlled clinical trial included 260 women with unexplained infertility undergoing IUI cycles. Patients were randomly assigned to receive either piroxicam 10 mg/day on days 4–6 after IUI or placebo (control group). The main outcome measures were number of IUI cycles, pregnancy, abortion, and multiple pregnancy rates. The pregnancy rate was found to be 25 (19.2%) and 16 (12.3%) in piroxicam and control groups, respectively ($p = 0.039$). Five patients (3.8%) in piroxicam group experienced twin pregnancy, whereas only three patients (2.3%) in control group had twin pregnancy ($p = 0.361$). The pregnancy rate per cycle was also significantly higher in those who received piroxicam as compared to controls (11.16 vs. 6.66; $p = 0.021$). Administration of piroxicam after IUI is associated with decreased number of cycles, as well as increased pregnancy rate and pregnancy rate per cycle in IUI cycles. However, piroxicam did not have any effect on abortion, multiple pregnancy, and ongoing pregnancy rates [139].

30.11 Conclusions

IUI is a simple, cost-effective, noninvasive first-line therapy for cervical factor, anovulatory infertility, moderate male factor, unexplained infertility, and immunological infertility with CPRs ranging from 10% to 20%. Optimal pregnancy rates following COH with a low-dose gonadotropin protocol and IUI may be obtained in couples with a short duration of infertility where the woman's age is ≤35 years with a PTMS count ≥10 × 106 and a 24-h survival of ≥70%, normal sperm morphology >4% (according to Kruger's criteria), 2–3 follicles ≥16 mm with uniformly high-grade vascularity and E2 levels >500 pg/mL on the day of hCG administration, adequate endometrium with a trilaminar pattern during insemination, and appropriately timed insemination using a soft atraumatic catheter. Strict patient selection criteria and individualized stimulation protocols tailored according to the age and etiology of the patient with a strict cycle cancelation policy will help to reduce the associated complications, such as multiple pregnancies and OHSS, while maximizing the overall pregnancy outcome. Three to six IUI cycles may be offered before considering alternate therapy. However, patients with advanced maternal age, severe male factor infertility, tubal pathology, or severe endometriosis will benefit from a direct referral to IVF/ICSI. Couples should be fully informed about the risks of IUI and COH as well as alternative treatment options. Larger randomized prospective controlled trials, investigating the important outcomes including live births, multiple pregnancies, miscarriage rates, and risk of ovarian hyperstimulation following IUI, are warranted.

Review Questions

1. How do you select a patient for IUI?
2. How will you work up the patient couple for IUI?
3. What will be the stimulation protocol you would select for your patient in an IUI cycle?
4. What is the correct technique of sperm preparation for IUI?
5. Would you prefer a blind IUI or an ultrasound-guided IUI?
6. Are there any known complications of IUI?
7. Are there any recent advances in the practice of intrauterine insemination?

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Conventional IVF Insemination

Michael L. Reed

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Learning Objectives

- Identify key environmental elements for developing a sperm capacitation protocol.
- Develop an understanding of the decision process regarding determining appropriate numbers of sperm for oocyte insemination.
- Identify key events from semen collection to sperm processing for insemination of cumulus-oocyte complexes.
- Identify a time line of events from oocyte retrieval to oocyte processing for insemination.
- Develop a knowledge base, including the historical perspective, for identification of physiological failure points for quality assurance and troubleshooting.

31

31.1 Introduction

In vitro fertilization (IVF) began by using what we call today conventional or traditional insemination techniques. In its most simplistic form, isolated cumulus-enclosed oocytes (in vitro matured or in vivo matured) are co-incubated with a number of motile spermatozoa (isolated from semen) for defined or less well-defined time periods. After co-incubation, oocytes are examined for proof that sperm penetrated the zona pellucida and oolemma: fertilization events that culminate in (1) formation of pronuclei that may be visualized in the oocyte cytoplasm or (2) cellular division if oocytes are evaluated after presumed pronuclear syngamy and dissolution.

On the surface, this approach appears to be very simple and not very challenging from a technical standpoint; in fact, achieving success with human in vitro fertilization is less challenging than working with a number of other mammalian species. Be that as it may, successful fertilization cannot occur in vitro without significant oocyte- and sperm-related events. Oocyte maturation involves resumption of meiosis (nuclear maturation) and completion of cytoplasmic activities, including production, accumulation, and activation of cytoplasmic components that allow nuclear maturation and support post-fertilization events (cytoplasmic maturation). Sperm maturation involves physiological changes that must occur during transit from the testicular environment to being housed within the epididymis prior to ejaculatory expulsion, which lead to fertilizing competence (capacitation) when the sperm approaches and binds to the oocyte zona pellucida.

31.2 Historical Perspectives

The first half of the twentieth century was explosive for reproductive physiology; endocrinology, ovarian physiology, and oocyte maturation and activation experiments were prominent; the fear that science would promote and forward a human-derived evolution was also common. Embryo transfer – movement of developing embryos, retrieved from a mated donor animal and then placed into the uterus or oviduct of a recipient animal – had been in practice since the late 1800s and further fueled concerns for humanity in this post-industrialized world.

In fact, the very real science that inspired the first chapter in *Brave New World* [1] allowed the public to experience a startling look into the basic principles of IVF and the possibilities for genetic manipulation (see [2]). But despite the many years of work dedicated to unraveling IVF, a basic understanding of the structural/functional changes that accompany sperm capacitation was lagging, and successful IVF in any mammalian species eluded researchers for decades, or was not substantially well-documented (see [2, 3]), including the many IVF attempts using human oocytes and sperm.

Regarding the practice of classical embryo transfer in humans, several publications, as early as in the 1980s and continuing into the early 1990s, described the attempts and successful utilization of embryo transfer with human oocyte donors – recovering uterine-stage embryos non-surgically (uterine lavage) from an oocyte donor that had been inseminated with the sperm from the husband of the embryo recipient. Despite the successes, this approach was eventually discontinued in favor of IVF [4]. Also interesting are the several photographs of human uterine-stage embryos presented in these multiple publications; embryos were photographed following lavage, but they were not studied to any extent prior to embryo transfer – notice that morphological features are very different from what we see in the laboratory today.

As one example of the early attempts with human in vitro fertilization, a large number of experiments were described, spanning a 6-year period. Rock and Menkin [5] described what is purported to the earliest account of successful fertilization in vitro following the observation of cleaving ova after insemination; 3 of 138 ova that had been exposed to sperm were cleaved. The time of evaluation was approximately 41–47 h post-insemination, but notably without evaluation for pronuclear formation events. Ejaculate sperm were washed in Locke's solution and then placed with oocytes at room temperature for 1 h (co-incubation also occurred in Locke's solution). There were no details regarding the sperm washing procedure, duration of sperm incubation in Locke's solution post-wash, or insemination concentration. Oocytes used in the Rock experiments were apparently retrieved from ovarian follicles during surgery, rinsed in Locke's solution, and matured in patient serum for 22 or 27 h prior to insemination. It is possible that serum components were carried over during co-incubation. Also consider the medium formulation used in these experiments: Locke's solution, per deciliter H_2O: 0.9 g NaCl, 0.042 g KCl, 0.024 g $CaCl_2$, 0.05 g $NaHCO_3$, 0.02 g $MgCl_2$, and 0.05 g dextrose. There will be more on the topic of capacitation media later.

Two landmark publications by independent researchers, Collin Russell Austin and Min Chueh Chang, cited the discovery that fertilization in vivo is preceded by a requisite exposure of sperm to the reproductive tract for several hours [6, 7]; capacitation, the term coined by Austin, refers to the many physiological and structural changes that sperm must

undergo to achieve the ability to undergo the acrosome reaction and fertilize an oocyte. Of interest, John Rock had been encouraged to apply the findings of Austin and Chang to his research, to repeat his experiments with in vivo capacitated sperm. However, Rock had already discontinued his work along these lines (see [2]), and there were no further attempts with IVF.

The discovery that sperm were required to undergo capacitation in vivo prior to fertilization led to the first successful IVF series; rabbit oocytes were exposed to sperm retrieved from the uteri of mated females. But fertilization of oocytes in vitro, with sperm not exposed to the reproductive tract but capacitated entirely in vitro, was not achieved until Yanagimachi and Chang [8, 9] were able to substantially document successful IVF in hamsters using epididymal sperm capacitated in vitro, where sperm were required several hours of incubation in vitro before any sperm-oocyte penetration events were noted.

The first well-documented, successful fertilization of human oocytes in vitro, using in vitro capacitated sperm, was reported in 1969 [10, 11]. The events specifically related to laboratory techniques leading up to this accomplishment were described in a detailed narrative by Barry Bavister [12]. Most notably, these successes were preceded by the significant foundation work set down by Dr. Bavister while he was a graduate student, studying golden hamster sperm capacitation.

31.3 A Conversation with Dr. Barry D. Bavister

I have had the pleasure of speaking with Dr. Bavister on several occasions regarding his work with sperm capacitation and IVF in the hamster model that led to the first successful human IVF – from these discussions and from his publications, I was able to build an image of the events, principal players, and emotions surrounding the successful fertilization as it played out, though I understand that it is difficult to convey the enthusiasm, the uncertainties, and the emotions in writing. I also posed specific questions to him about the timing of the events, the conditions for sperm capacitation, and the decision to use 1×10^6 million/ml sperm for the co-incubation.

In 1968, Barry Bavister was a graduate student working on hamster IVF; he focused on sperm requirements and refining the hamster IVF technique that had proven successful for Yanagimachi and Chang [8, 9]. He was approached by Dr. Edwards and given the opportunity to apply his experiences and advances with the hamster model (see below) to the problem of human in vitro fertilization. In context, Dr. Edwards had been working on oocyte maturation (including human oocytes) for several years [13, 14], but as with other researches documented, successful fertilization was lacking. The basic approach taken by Dr. Edwards then was to re-examine human IVF by building upon the success that Dr. Bavister had achieved with hamster IVF, using the modified culture medium that had proven successful for hamster sperm capacitation and gamete co-incubation.

To obtain sperm, a hamster epididymis is punctured while it is submerged in culture medium; the dense exudate passes into the culture medium; numbers of sperm (live and dead) released from each preparation can be extremely variable. Hamster IVF results were also variable, leading Dr. Bavister to question co-incubation conditions; one aspect, culture medium pH, was evaluated via qualitative observation (phenol red was added to the medium for comparisons against color controls). Dr. Bavister noticed that pH varied between the different sperm preparations, and when motile (viable) sperm numbers were higher after co-incubation, fertilization rates were lower, and when motile sperm numbers were lower (dead and nonmotile), fertilization rates were higher. The link between viable sperm concentration and pH was realized, where pH was altered according to numbers of viable sperm, or more specifically, by the concomitant concentration of metabolic acids produced by the sperm. Patterns of fertilization – monospermic, dispermic, and polyspermic penetration – were also correlated to medium pH (see [12] for more details). And fortunately, the acrosome reaction in the hamster could be visualized without staining; Dr. Bavister noted that the proportion of acrosome-reacted sperm increased as pH became more alkaline. In subsequent trials, controlling for both medium pH (adjusting bicarbonate and CO_2 to target pH of 7.6) and viable sperm concentration yielded the highest fertilization rates with minimized polyspermic fertilization. Dr. Bavister discovered and then followed up on key elements leading to successful and consistent sperm capacitation in vitro.

The gamete co-incubation system chosen for the human IVF trials was Tyrode-B [Tyrode's medium modified to 32.6 mM bicarbonate, 2.5 mg/ml bovine albumin, 0.1 mM pyruvate; 5% CO_2/air at 37 °C to maintain an alkaline pH (pH 7.6)]; a sperm concentration of 1×10^6 million sperm per ml, based on experiences with hamster sperm concentration and pH, was chosen. Human sperm were washed from the ejaculate and capacitated for 6–7 h prior to being co-incubated with in vitro matured oocytes; and each trial, by necessity, utilized only a few human oocytes, as starting materials – surgically retrieved ovarian tissue – were scarce. After multiple attempts, evidence of fertilization was finally observed, culminating in two remarkable publications [10, 11]. Human sperm capacitation times in vitro had not yet been fully defined despite progress made by Dr. Bavister; but based on the work of Chang [7], Austin [6], and Yanagimachi and Chang [8, 9], the in vitro incubation of human sperm for 6–7 h was thought to be acceptable, to achieve capacitation for the human IVF trials. The first successful fertilization events occurred with in vitro matured oocytes: (1) of the 56 oocytes held in vitro, 34 underwent nuclear maturation, and (2) of the 34 oocytes inseminated in this trial, many displayed evidence of zona binding/penetration, while only 2 were confirmed with 2 pronuclei. But, the birth of the first IVF baby is not achieved until much later, and only after IVF with oocytes that had been matured in vivo and recovered

surgically [15]. Compare Locke's solution (discussed earlier) to the Tyrode-B medium formulations and sperm incubation conditions, but also consider how this narrative relates to the present-day knowledge base regarding human sperm capacitation conditions: medium pH, sperm pHi, elevated bicarbonate, albumin, temperature, and time.

31.4 The General Process of IVF: Conventional Insemination

Retrieval of oocytes has moved, for the most part, to recovery of in vivo matured follicular oocytes via ultrasound-guided needle aspiration. The earliest successful pregnancy [11] utilized a surgical approach to obtain in vivo matured oocytes, as pregnancies, despite fertilization in vitro, had not been obtained using in vitro matured oocytes, an early demonstration of the functional difference between oocytes that develop and mature in vivo, compared to in vitro (see [16]). Ovarian stimulation by injection of gonadotropin preparations is the most common treatment prior to oocyte recovery, though minimal stimulation and natural cycle follicle aspiration cycles are also offered. As the purpose of this chapter is to review conventional IVF, ovarian stimulation protocols and oocyte aspiration procedures will be left to others for review; it is sufficient at this point to note that human ovaries are not poly-ovulatory in nature; rather it is more common that a single follicle matures for ovulation.

Follicle aspiration is usually preceded by an injection of a chorionic gonadotropin preparation with LH activity and/or injection of LH or GnRH; 34–36 h later (prior to ovulation), oocytes are recovered by insertion of the aspiration needle through the vaginal wall and into the ovarian follicular structure(s). Follicles are drained by application of vacuum pressure, and aspirates are then handed off to the laboratory for evaluation. At this stage, the cumulus cells surrounding the oocyte are visibly "watery," or "clear" with individual or clumps of cumulus cells suspended in an expanded hyaluronic mucin matrix. These complexes (cumulus-oocyte complex (COC)) are rinsed to remove blood and minimize carry-over of follicular fluid – which contains a number of serum exudates, which may or may not include anesthetic agents [17], toxins [18], or microorganisms [19–22]. The COCs are then placed into culture medium for several hours until co-incubation with sperm. The cumulus cell hyaluronate complex surrounding the aspirated oocyte may be quite large in relation to the size of the oocyte, and there may be small blood clots, degenerated cells, or follicular granulosa cell sheets attached; excess cumulus may be trimmed prior to insemination. And while cumulus may be removed prior to conventional insemination [23], as much as is done with sperm injection cycles, fertilization may not occur as efficiently as with insemination of intact COCs; cumulus cells and the surrounding hyaluronate matrix play a role in sperm preparation for fertilization and may be involved with sperm chemotaxis, via cumulus cell secretion of progesterone [24, 25].

Ejaculate sperm are processed out of the semen sample by one of the several techniques [26]. Two of the more common methods are density gradient separation and swim-up. The motile population of sperm is "enriched" by these preparations, via reduction of the majority of motile, less motile, and nonmotile sperm and non-sperm cells. The enriched population of motile sperm is suspended in capacitation medium and incubated for several hours prior to co-incubation with COCs.

A protocol for co-incubation of sperm and oocytes is usually formatted to define a specific number of motile sperm per oocyte per volume of medium, e.g., 50,000 sperm per oocyte/ml. Protocols differ from laboratory to laboratory, where sperm concentrations may range from 20,000 to 1×10^6 motile sperm/oocyte/ml. Extremes in sperm concentration will depend more so on the co-incubation vessel – well dish, micro- or macro-drops of medium under oil, test tubes, or other vessels – as the choice of vessel can dictate proximity of sperm to the COC. For example, a cell-well dish with 1 ml medium and one oocyte would require more sperm per volume than a 100 ul drop, under oil, that contains one oocyte; specifically, sperm in the micro-drop under oil are physically confined in closer proximity to the oocyte, and random encounters between sperm and oocyte investments are more likely. In any case, co-incubation may be allowed to continue for several hours to overnight – but evaluation for fertilization typically begins 16–18 h, or a similar interval from the time co-incubation was initiated. Pronuclear formation may be optimal within this time-frame, though earlier and later pronuclear formation is not uncommon. Ova are separated after evaluation, e.g., degenerated, immature, unfertilized, mono-pronuclear, bi-pronuclear, and tri-pronuclear and greater pronuclear, and may also be sorted based on morphology. Culture proceeds per laboratory protocol, with evaluation of development at defined intervals, prior to transfer, cryopreservation, or manipulation for genetic testing.

When patients refuse to use sperm injection despite having limited numbers of motile sperm in the ejaculate, it may be possible to bank sperm via cryopreservation of numerous ejaculates, or in cases where a few ejaculates would be sufficient, a multiple ejaculate collection protocol may be employed [27]. Insemination of COCs may also be approached, in cases with limited numbers of motile sperm, by insemination of COCs across a defined time interval, for example, using 1/3 of the capacitated sperm at each of three 1 h intervals; COCs may have to be combined into several or a single large macro-drop or test tube for this approach to work (Reed, unpublished data).

31.5 The Oocyte

Oocyte sourcing is, of course, ovarian – but the intermediate step(s) toward insemination include in vivo maturation, in vitro maturation, and use of cryopreserved oocytes. The use of sperm injection is recommended following cryopreservation of oocytes – ultrastructural changes impact

sperm/zona/oolemma dynamics with conventional insemination, so that topic will not be addressed [28–30]. In vitro maturation (IVM) has been practiced now for several years; the processes and protocols for IVM, in brief, typically rely on ovarian stimulation protocols with or without a priming treatment (chorionic gonadotropin, or LH/GnRH) prior to aspiration. A key difference is that ovarian follicles are aspirated at a much smaller mean diameter, germinal vesicle stage, and pre-cumulus expansion, followed by a period of in vitro culture (24–48 h) for nuclear and cytoplasmic maturation prior to insemination. These oocytes may respond equally, regarding post-insemination developmental and pregnancy potential, but there are differences in cytoplasmic maturation that may influence the outcomes (see [31, 32]). Insemination of IVM oocytes occurs in the same manner as oocytes retrieved just prior to ovulation: conventional insemination or sperm injection [33].

Conventional insemination protocols may differ on timing of insemination, according to the perception of oocyte maturity. Cumulus-oocyte complexes can be scored according to the expansion of both outer cumulus and the specialized inner cortical, zona-proximal granulosa cells (corona radiata). Immature, mature, and post-mature are used to describe COCs in some cases. Insemination times may be delayed for COCs that appear to be immature, though most protocols call for insemination of all COCs at the same relative time. Density of the cumulus and coronal cells may prohibit a true visualization of the oocyte cytoplasm and also make it difficult to visualize polar body structure(s).

There are two techniques, anecdotal but not practiced in my laboratory, used to displace the cumulus and mucin matrix, which results in a flattening of the COC to allow visualization:

1. Placement of a COC in a very small amount of medium, onto the bottom of a sterile Petri dish; the dish is slapped down onto the microscope stage, spreading the cumulus and mucin matrix out around the oocyte – this technique can allow for visualization of cytoplasm and polar body structure(s).
2. Drawing the COC into a small-diameter glass Pasteur pipette, essentially elongating the COC so that the oocyte can be viewed through the wall of the pipette.

The information gained by these two techniques may be overshadowed by the potential to compromise the oocyte via mechanical, pH, and temperature stressors. Qualitative evaluation of cumulus expansion may be useful (but should not be considered to be a reliable predictor of nuclear maturation) and is used by some laboratorians to assign insemination times. The laboratory generally has to rely on receipt of oocytes "as is," with regard to maturation and quality. Evaluation of oocytes at the time of fertilization evaluation will be covered in another section. Further, oocyte quality is not something that can be easily and positively modified by the laboratory before insemination; as such, ovarian stimulation protocols will not be addressed, but for further reading, see Beall et al. [34], von Wolff et al. [35], and Jungheim et al. [36].

31.6 Oocyte Nuclear and Cytoplasmic Maturation

The final stages of oocyte growth include acquisition of competency to maturation. The oocyte is released from stasis following limitation of cumulus cell-to-cell contact and release of cumulus cell contact with the oocyte membrane; the specialized cells close to the oocyte communicate directly with the oocyte via projections that extend through the zona and into the oocyte. Compartmentalization – and isolation via gap junctions – of the oocyte via these specialized cells allows for participation in and control of oocyte growth and maturation. Prior to ovulation exposure to luteinizing hormone (LH), or in the event of mechanical aspiration (in most IVF cases), an exposure to LH, chorionic gonadotropin (hCG), and/or LH after gonadotropin-releasing hormone (GnRH) administration accelerates the communication breakdown between the cumulus cells and the oocyte.

The simplified explanation is that the cumulus-oocyte communication breakdown is receptor-mediated, at the level of the cumulus cell – and withdrawal of the coronal cell projections from the oocyte membrane leads to a decrease in transport of inhibitory molecules from the cumulus to the oocyte. This leads to release from meiotic stasis, meiosis resumes, and one-half of the oocyte chromosome complement should be released or extruded from the oocyte in the form of the first polar body; essentially this process can be viewed as an unequal cytoplasmic division, where the oocyte proper contains a 2 N complement and the polar body contains a 2 N complement resulting in a metaphase II oocyte. Nuclear maturation does not imply that cytoplasmic maturation has occurred, but rather cytoplasmic maturation has proceeded to a set point where nuclear maturation is possible. Removing an oocyte from the follicular environment (and follicular fluid) may trigger spontaneous nuclear maturation (see [37, 38]), without cytoplasmic maturation.

Unlike polar body extrusion, acquisition of cytoplasmic maturation and future developmental competence is not a tangible event [39]. During growth and final stages of maturation, the oocyte builds a reservoir of maternal mRNA transcripts, cytoplasmic growth factors, and enzymes for processing the sperm (decondensation, remodeling, and pronuclear formation) and for pre- and post-syngamy events including cellular division. Essentially, the oocyte and early cleavage-stage embryo operate off of maternal controls up to the point of comprehensive genomic activation. Failure to achieve cytoplasmic, or molecular maturation as it has also been called, can lead to fertilization and post-fertilization event failures regardless of the method used for insemination of the oocyte [40].

31.7 The Sperm

There are two sources for sperm that do not require surgical intervention: antegrade and retrograde ejaculate. There are three primary sources for sperm that do require surgical intervention: seminal vesicle, epididymal, and testicular.

A less-often used source for sperm is recovery from the vas deferens at time of vasectomy or vasectomy reversal. As testicular sperm are immature and fewer in number compared to ejaculate sperm, sperm injection is the method of choice for assisted fertilization – this source will not be discussed in detail here; however, sperm may be harvested from tubules "fresh" at the time of retrieval, and/or they may be cryopreserved for future use. Seminal vesicle sperm aspiration can be successful in patients with ejaculatory obstruction, but despite recovery of very good numbers of sperm, the quality of the sperm retrieved from these structures may be compromised by the physiology of the seminal vesicle environment and of course the backlog of sperm due to ejaculatory blockage; sperm aspirated from seminal vesicles are characterized by very low motility and increased sperm DNA fragmentation (Reed, unpublished), making these sperm candidates for sperm injection procedures rather than conventional insemination [41–44].

31.7.1 Epididymal Sperm

The epididymis is situated outside of the testicular environment, with four associated segments: caput, corpus, cauda, and the proximal vas deferens. Epididymal aspiration is accomplished by percutaneous or microsurgical aspiration (see [45]). A complete review of the biology of the epididymis is beyond the scope of this chapter; however, it should be recognized that in the context of sperm fertilizing ability, particularly in reference to conventional insemination, transit of sperm through the epididymis is critical to final maturation of sperm – the ability to achieve progressive motility and sperm membrane modifications that allow sperm to undergo capacitation and the acrosome reaction – these are hallmark events critical for in vivo and conventional in vitro fertilization. It is also important to keep in mind that the epididymal environment may be modified following vasectomy reversal or other injuries/pathologies. For reviews on epididymal anatomy and functions, see Cooper [46–48], Cornwall [49], and Hinton [50].

In many species, epididymal sperm can be used for conventional in vitro fertilization (see Brackett [51]), and it is reasonable to consider that human epididymal sperm may also be used for conventional insemination; however, early successes became historical footnotes after widespread application of sperm injection techniques to human IVF.

In 1988, Silber et al. [52] retrieved epididymal sperm from two patients with congenital absence of the vas deferens; sperm were recovered from the proximal epididymis in both cases and used for conventional IVF preceding ZIFT. Aspirated sperm were diluted in modified Tyrode's with serum albumin; motile sperm were separated using a swim-up technique. Sperm were capacitated for 1 h in the modified Tyrode's and then aliquoted to tubes containing oocytes (5–7 per culture tube; Menezo's B2 with heat-inactivated fetal cord serum). Couple 1 yielded 28 oocytes, and all oocytes were exposed to sperm; 16 of 28 oocytes were fertilized. Couple 2 yielded 24 oocytes, and all oocytes were exposed to sperm; 6 of 24 oocytes were fertilized. One clinical and one biochemical pregnancy resulted. Also, Hirsh et al. [53] found that motility of the epididymal sperm was highest if taken from the caput, in 89% of patients, but overall despite differences in sperm motility by region, the mean fertilization rate following conventional insemination was only 11.2% and an overall 8.7% implantation rate per embryo.

One of the largest studies published describing outcomes after conventional insemination with epididymal sperm also included a smaller cohort of patients that underwent sperm injection using epididymal sperm [54]. From the publication, it is apparent that the two treatments were independent, that is, sibling oocytes were not divided within patient to explore conventional vs. sperm injection outcomes. Details regarding capacitation or other sperm incubation conditions were not presented; however, in this case, the data clearly demonstrated that the outcomes, both fertilization and gestational, were superior following sperm injection with epididymal sperm. Of 1427 oocytes undergoing conventional insemination, 98 embryos were generated, with an ongoing pregnancy rate of 4.5%, compared to sperm injection with 197 oocytes yielding 80 embryos and an ongoing pregnancy rate of 30%.

31.7.2 Ejaculate Sperm

Ejaculate sperm are recovered from semen using one of the many techniques (see Henkel [26]), where the goal is to concentrate or isolate motile sperm from the population of sperm and non-sperm cells in the ejaculate. Discontinuous gradient and swim-up are two of the most common methods used, but like many techniques, efficiency of motile sperm recovery varies from specimen to specimen. Sperm cell separation appears to be a simple process, on the surface, but extreme care must be taken during the procedures, to maintain aseptic technique and to prevent carry-over of contaminants from the semen that might inhibit sperm capacitation [55].

In some cases, it may be advisable to collect more than one specimen [27] for processing or to cryopreserved sperm ahead of the oocyte retrieval day as physiological and psychological pressure may make semen collection on the day of oocyte retrieval difficult.

31.8 Sperm Capacitation

Human ejaculate sperm are usually simple to capacitate, but details regarding incubation environment are still important to maximize the number of sperm that can undergo capacitation: (1) pH, (2) bicarbonate, (3) protein, (4) calcium, (5) temperature, and (6) time. Capacitation is a term used to describe a complex series of events that must occur before the sperm cell acquires the ability to fertilize an oocyte. As capacitation proceeds, the sperm gain the ability to hyperactivate; and these are the sperm that have the greater potential for undergoing the acrosome reaction [56].

While it seems simple on the surface, sperm biochemistry is a complex area of andrology: sperm membrane modification and hyperpolarization, intracellular ion shifts, protein tyrosine phosphorylation, and reactive oxygen species. I would point the reader to a more comprehensive series of papers for study [57–64].

An example of a simple sperm capacitation protocol would be to separate sperm (density gradient or swim-up) and concentrate the motile sperm by centrifugation. Resuspend the sperm at a concentration of 10 m/ml motile sperm in HTF with 10 mg/ml albumin at pH 7.5, and incubate at 37 °C for 4 h. At the time designated for oocyte insemination, aliquot an appropriate number of motile sperm into equilibrated medium (the same used for the oocytes), and place sperm with the COCs.

Most commercial media today are manufactured to achieve a given pH range at a given concentration of CO_2. Look closely at the manufacturer Certificate of Analysis; if there are any doubts, consult the manufacturer. The current thinking for embryo culture conditions is to maintain a lower pH relative to ideal pH for capacitation of human sperm, for example, embryo culture is targeted at pH of 7.2 in some laboratories and 7.3 in others [65]. Acrosome function, like motility following capacitation is pH dependent, and internal pH can be modified according to extracellular pH; but importantly, pHi should increase as capacitation proceeds. Under progesterone-challenge conditions, sperm acrosome responsiveness was improved as external pH increased [66]. Sperm ion channel regulation then is critical; and the only externally visible metric is modification of motility characteristics that change with in vivo and in vitro environmental pH [67–69]. These ion channels are also responsible for intracellular rise in calcium [70, 71]. Monitoring pH is, with regard to sperm cells, an important concept to remember as capacitation may not be efficient, or it may not occur at all for some patients, resulting in lower than expected fertilization rates. An anecdotal lesson from my own laboratory: on receipt of a "new-generation" culture medium many years ago, fertilization rates fell consistently to less than 10% across several patients. This same medium was used for both sperm capacitation and embryo culture, and medium pH was targeted to 7.18; changing to a different medium for sperm capacitation, targeted to pH 7.5, immediately solved the problem. Other laboratories expressed similar experiences.

Bicarbonate is critical for capacitation, both in vivo and in vitro [72–74]; and while culture media formulations vary [75], the concentration of bicarbonate in commercial culture media is sufficient for capacitation to occur, these concentrations being similar to if slightly lower than found in vivo. And of course, bicarbonate is a key player in maintenance of pH. A note of caution regarding bicarbonate: it is acceptable to incubate sperm destined for sperm injection in modified media, e.g., Brand-X with HEPES, or MOPS, or HEPES and MOPS, but these media are designed for use "in air," and not with incubator concentrations of CO_2. Most of these media have approximately a fivefold lower concentration of bicarbonate; the only modified medium that should be considered for sperm capacitation should have adequate bicarbonate as well, e.g., 25 mM bicarbonate, in addition to the Good's buffers [76, 77].

The type of protein supplementation is important for sperm function [74]; albumin is recognized as important for sperm membrane modifications and energy store regulation. Would inadvertent use of a medium, not supplemented with a protein source, affect capacitation efficiency? One study [78] trialed a sperm capacitation medium, SMART1®, which, "based on Earle's salts solution, contains glucose, amino acids, synthetic and plant molecules and is devoid of any animal compound." Initial capacitation trials and acrosome reaction challenges demonstrated reduced sperm movement (kinematics) characteristics, but similar acrosome reaction profiles. IVF using sibling oocytes demonstrated a significant increase in oocyte fertilization after using SMART1® for both capacitation and sperm-oocyte co-incubation, compared to the medium with albumin: 65% vs. 55%, respectively. There were no significant differences in the quality of embryos or transfer outcomes. A companion paper [79] compared SMART2®, a culture medium "devoid of any human or animal compound" for culture following sperm injection; no differences between treatments were found for embryo morphology, but pregnancy outcomes could not be compared. And last, comparing post-gradient motility characteristics between two media with albumin to protein-free (PF) medium (containing methylcellulose), it was noted that differences observed between treatments, e.g., lower percentage progressive motility and reduced sperm survival in PF medium, might be attributed to sperm sticking to the analysis chamber surface in PF medium; there was an increased sperm yield with PF medium, and there were no differences for washed sperm morphology or 24 h survival. No attempts were made to test the functionality of the sperm, e.g., binding or fertilization [80].

Use of albumin and albumin/globulin supplementation is most common, while use of heat-inactivated patient or fetal cord serum has essentially been abandoned. Media are typically available supplemented with 4, 5, or 10 mg/ml albumin and are acceptable to incubate sperm. Note also that one protein supplement, in particular, Synthetic Serum Substitute (Irvine Scientific, Irvine, CA), is formulated using saline as a base, rather than a bicarbonate base medium; therefore, pH will be slightly more acidic due to dilution of the bicarbonate when adding this supplement to protein-free medium. Also, a protein supplement and culture medium formulation interaction may exist, potentially reducing capacitation efficiency; medium selection must be evaluated independently [81], though this effect might be due to contamination of a protein supplement by a stabilizer common in some commercial preparations [82] and would require further study. Proteins, e.g., albumin particularly, not only participate in sperm membrane modifications during capacitation, but they also provide modest oncotic pressure, and they participate in surface particle charges, preventing sperm, ova, and embryos from sticking directly

to glass and plastic pipettes and surfaces. Other molecules, e.g., PVA and methylcellulose, may substitute for some of these nonphysiological functions.

Limiting temperature excursions should be a standard practice in any IVF laboratory for all aspects of the process: oocyte retrieval and identification of COCs, rinsing, and incubation (pre-, co-incubation, post-fertilization). Incubator and surface temperatures must be defined and rigorously monitored.

The temperature for sperm capacitation should not exceed the temperature limits set for embryo culture. Cooler temperatures, e.g., room temperature, may be sufficient, but maximizing sperm function for reliable and consistent results should be the goal; room temperature may also be different between laboratories. Time is one area of argument; how long must sperm be incubated to achieve optimal capacitation? The early success achieved by Barry Bavister in animal and human IVF demonstrated that time was also important; sperm were incubated for 6–7 h at 37 °C prior to insemination. But remember that sperm do not immediately bind to and penetrate the oocyte vestments; exposing sperm to oocytes does not stop capacitation; rather it occurs across a continuum, where different subpopulations will achieve capacitation at different times, including the time of oocyte-sperm co-incubation. The time set aside for sperm capacitation prior to co-incubation may be more important for the "short co-incubation" protocols that minimize sperm-oocyte co-incubation, e.g., where the protocol calls for rinsing oocytes after 1 or 2 h.

Consider maintaining a separate culture incubator for sperm capacitation; simple media, e.g., one of the HTF versions, with at least 4 mg/ml albumin and 25 mM bicarbonate, maintained to target pH 7.5 provide a consistent environment for incubating sperm. This is also a good technique to troubleshoot changes in overall fertilization rates. Remember that incubation of very large numbers of motile sperm per volume of medium will effectively reduce pH over time and may result in inhibition of sperm capacitation; see the notes from my discussions with Barry Bavister as an example. This is a simple experiment that can be done in any laboratory; begin with equilibrated medium that has a phenol red indicator; resuspend motile sperm to large-step concentrations, e.g., 1, 10, and 100 m/ml, by adding more sperm or reducing the volume of medium. Incubate for several hours, and then compare the color of each tube to each other.

31.9 Modification or Enhancement of Sperm Function Prior to Insemination

Sperm may be treated with various factors, hormones, and buffers in an attempt to improve sperm function prior to insemination (see [83]). Modifiers of motility (and therefore capacitation and acrosome reaction), for example, include exposure to heat-inactivated patient follicular fluid; progesterone; cAMP modifiers such as caffeine, pentoxifylline, or theophylline [84]; and growth factors such as platelet-activating factor. Extenders formulated originally for nonhuman animal sperm may also be useful; egg-yolk zwitterion buffers can maintain sperm function for at least 24 h and may help preserve sperm membrane integrity.

31.10 Oocyte-Sperm Co-incubation: The Devices

Culture tubes, well dishes, and micro- or macro-drops of medium under oil all have been used for conventional insemination of oocytes. Each device has both good and bad characteristics that should be considered, in particular, when trying to troubleshoot fertilization failure patterns, increased polyspermic fertilization rates, and so on. Selecting one device over another should include a review of the ease of use, e.g., placement of oocytes prior to insemination and recovery of ova for evaluation after co-incubation, and sperm behavior in the device.

Well dishes are typically large volume (usually 0.25–1.0 ml wells) with sloped or straight walls, though in recent years, there are a number of newer dishes with small-volume wells. Larger volumes used for co-incubation require larger numbers of motile sperm. For example, a center well dish with 1.0 ml medium containing one COC could be inseminated with 10^6 motile sperm; a case with ten oocytes would require ten separate dishes and 10×10^6 motile sperm under this configuration. More than one COC may be placed together for insemination. The dishes may be used with or without an oil overlay, but as with any culture dish used without oil, proper humidity must be maintained to minimize osmotic elevation due to evaporation of medium. Sperm will migrate outward to all regions of the well, and if deep enough, sperm will migrate upward as well. The COC and the oocyte after co-incubation can be visualized more easily, though straight-wall wells may be harder to use as ova may have moved to the edges against the wall where the visual image is less well-defined. Round and sloped wells are more easy to use.

Culture tubes, e.g., 4.0 ml round-bottom polystyrene culture tubes, are also common for co-incubation (and embryo culture in some labs) and may be used with or without oil. For example, 1.5 ml medium with a single (or more) COC may be inseminated with a lower number of motile sperm compared to a large-well dish; the physical shape of the culture tube, despite the volume, will maintain a greater concentration of sperm at the lower limits of the tube, though migration upward will still occur. Placing oocytes into culture tubes is relatively simple, but retrieving them after co-incubation requires either pipetting or pouring out; both techniques risk loss of the oocyte(s), and repeated rinsing may be needed to dislodge the ova. Pipette tips should be fire-polished to minimize scratching or etching of the plastic during the placement or retrieval of the ova.

Micro- and macro-drops under oil are efficient devices or containers for co-incubation; multiple drops per dish make management easier and effectively reduce the number of motile sperm required for co-incubation due to the physical limitations of the drop. For example, if the protocol calls for 10^5 motile sperm/oocyte/ml and the volume of the drop is 100 ul, then 10,000 motile sperm per oocyte per drop are required. The drop is shaped like a half-dome, contained by the oil overlay. COCs are easily placed, and ova are easily recovered after co-incubation as the entire drop can be visualized at one time.

There are a number of devices and dishes to consider for fertilization and embryo culture; please see [85, 86]. And regarding the topic of oil overlay, consider that one benefit of an oil overlay, aside from osmotic control, is that the oil behaves as a buffer between the medium and the outer environment. Case in point: a QA evaluation of sudden depression of fertilization rates determined that co-incubation of COCs and sperm in culture tubes without oil overlay exposed the culture medium directly to poor-quality air in the laboratory [87].

31.11 Insemination Concentration, Timing, and Duration of Gamete Co-incubation

Many of the protocols for conventional insemination, with regard to sperm concentration, need to be evaluated according to the device/dish used for insemination; millions of sperm/ml/oocyte is a common nomenclature. There are few publications directly and experimentally addressing insemination concentration.

One early publication [88] evaluated sperm concentration and fertilization outcomes (normospermic and polyspermic); insemination was carried out in organ well dishes (0.9 ml medium), and the authors describe each oocyte as a unit of study. Insemination concentrations were 500,000, 100,000, 50,000, 25,000, and 10,000 motile sperm/ml. Fertilization rates (%) and total oocytes inseminated for each of these treatments were 60.0% of 65 oocytes, 60.9% of 146 oocytes, 75.5% of 351 oocytes, 80.8% of 26 oocytes, and 28.6% of 7 oocytes. The incidence of polyspermic fertilization decreased as sperm numbers decreased. The authors described optimal numbers of sperm for insemination, based on normospermic and polyspermic fertilization outcomes, under these culture conditions, to be between 25,000 and 50,000 motile sperm/ml.

Increasing the numbers of motile sperm/ml may be useful in some patients [88, 89], for example, where previous fertilization failure had occurred, or in men with reduced sperm morphology or other sperm pathologies that may affect sperm function [90]. There is the requirement for increased numbers of sperm for insemination in vitro compared to what is presumed to be happening in vivo; laboratories cannot compete with in vivo physiology in regard to processing and selection of sperm cells. And this may also be explained, in part, by the fact that in vitro, only a small percentage of sperm can actually capacitate, undergo the acrosome reaction, and bind to and penetrate the oocyte vestments [91, 92]. Anecdotally, before the widespread use of sperm injection, low to no fertilization was more common, loosely estimated at 20% or so of all conventional insemination cycles. Certainly morphology and other sperm-related issues played a greater role in this. As sperm injection gained acceptance, sibling oocyte studies demonstrated the efficacy of sperm injection vs. conventional fertilization, most notably for surgically retrieved sperm [54], and in patients who do not have "male factor" infertility, study results vary, showing improved, no, or slight benefits of one approach over the other (see [93–95]).

Timing of insemination is discussed in relation to oocyte retrieval or more appropriately in relation to the administration of hCG to simulate the LH surge. Pre-ovulatory oocytes are harvested, incubated in vitro, and then placed with motile sperm for co-incubation. One of the earliest studies [96] demonstrated that fertilization rates (and % cleaved embryos) improved if insemination was delayed relative to the time of oocyte retrieval. The study spanned 0–0.5 h post-retrieval, then 4–4.5 h post-retrieval, 5–5.5 h post-retrieval, and 6–6.5 h retrieval. Essentially, this study demonstrated that pre-ovulatory oocytes required time to finalize "ovulating" in vitro. The intervals studied, essentially 4–6 h post-retrieval for insemination, are still in use. However, the interval is not absolute, as sperm and oocyte interactions continue, as do capacitation and oocyte maturation; Jacobs et al. [97] found no significant differences, regarding fertilization and post-fertilization development for conventional fertilization (1–7 h post-retrieval) or sperm injection (0.5–8 h post-retrieval) across a large number of cycles. From a quality assurance standpoint, standardizing sperm capacitation times and insemination times is a good practice; assignment of duties and timing of events can help during troubleshooting and help manage a busy schedule.

Management of semen specimens on the day of oocyte retrieval may be assisted by having all semen specimens required for cases that day to be collected early in the day. Semen specimens may be extended, 1:1 v/v with Refrigeration Medium TYB (Irvine Scientific, Irvine, CA), cooled, and held until processing; use of this buffer also allows specimens to be collected and extended on the day prior to the oocyte retrieval [27].

Duration of sperm-oocyte co-incubation varies: short co-incubation to the more generic "overnight" incubation. Sperm and oocytes are placed together at some point during the day of retrieval and then allowed to incubate until fertilization determination. Consider that sperm and cumulus cells produce metabolic waste products; sperm in particular, due to the higher numbers required for consistent fertilization, can as discussed earlier modify medium pH. Short exposure protocols, e.g., as little as 1 h co-incubation, have been successful in some settings [98], demonstrating that sperm penetration of the cumulus/hyaluronate matrix and functional sperm/zona receptor binding can occur quickly. Comparing a 6–16 h co-incubation period, Xiong et al. [99]

found that all outcome parameters were not significantly different, with the exception of a significantly reduced polyspermic fertilization rate with the shorter co-incubation period. Note that in this study, a relatively large sperm concentration was used: 10,000–15,000 sperm in a 50 ul drop of medium.

Evaluation for fertilization, e.g., pronuclear formation, varies also from lab to lab, from evaluation at 14–18 h post-insemination as one example to a time specified as, generically, "early the next morning." Dedicated laboratorians arrive very early in the morning for adherence to strict time lines, and this may be more important if you are using a pronuclear grading system [100] or using some other mechanism to determine early predictive potential [101]. But for all practical purposes, evaluation for pronuclear formation can be done at slightly later intervals, though data regarding embryonic potential may be lost, as demonstrated by time-lapse monitoring of pronuclear dynamics [102].

31.12 Recovery and Stripping of Oocytes for Fertilization Determination

COCs that have been exposed to sperm cells undergo a gross morphological change; enzymatic activity from release of sperm acrosomal contents causes a digestion of the hyaluronate matrix suspending cumulus cells around the oocyte. A collapse of these cells and a condensation of coronal cells immediately surrounding the oocyte can make visualization of the cytoplasm difficult. Complete or partial removal of cells from around the oocyte using a mechanical technique is required in most cases, and for new embryologists, this can be one of the most frustrating techniques to master; losing an oocyte or breaking an oocyte will happen eventually. And depending on the device/dish used for co-incubation, oocytes may have to be moved to another device for fertilization determination, e.g., oocytes inseminated in round-bottom culture tubes must be pipetted or otherwise recovered from the tube(s) and placed into a dish with medium prior to further processing. Using a dissection microscope, the oocyte must be freed from cumulus and coronal cells. The two most common approaches are (1) use of small bore pipettes, circa 130–140 um to gently aspirate the oocyte from the cells, or (2) use of small gauge needles, e.g., 27.5 or 30 g, to tease and push the oocyte from the collapsed cells. A combination of these two techniques is also common. Pulled glass pipettes with sharp edges at the tip are still used in some labs, though bore diameter is critical; too small and the oocyte may rupture; too large and the cells may not be sufficiently removed for visualization. Commercial small bore pipette tips come in a variety of sizes and are convenient, but it is still important to visually verify the diameter of the pipette tip before use; mistakes in manufacturing are not common, but possible. Other methods, for example, pulling the oocyte gently into the bevel of a 27.5 g needle, are more difficult, but each embryologist will need to master at least one technique.

31.13 Evaluation of Fertilization

In basic terms, the goal of the fertilization determination is to verify presence (and number) or absence of pronuclei and, in the absence of evidence of fertilization, to determine oocyte nuclear maturation status and evaluate sperm/zona binding. The biology of sperm decondensation, remodeling, and pronuclear formation is complex and will not be considered in detail (see [103–106]). Also, the events marking oocyte activation at fertilization, stemming from sperm fusion or sperm injection, will be yielded to the reader; the complexities of cytoplasmic events, translation of cytoplasmic mRNA stores, and microstructural changes can be found in detail elsewhere (e.g., see [107]).

Pronuclear formation following fertilization is not expected to be in perfect synchrony, as oocyte maturity and sperm penetration timing varies, particularly with conventional insemination compared to sperm injection cycles that are more predictable in regard to oocyte nuclear maturation status and sperm injection timing [108–110]. The majority of ova that will display pronuclei can be evaluated between 16 and 18 h post-insemination; however, early cleavage can be observed in some ova, as early as 20 h post-insemination particularly following sperm injection.

Fertilization status, specifically in relation to pronuclear observation, includes monospermic (1 sperm) and polypronuclear (polyspermic and polygynic). Monospermic fertilization (normospermic; one sperm) is evidenced, classically, by the presence of two to three polar bodies and two pronuclear structures in the cytoplasm. Maternal and paternal centriole structures provide the foundation for pronuclear formation; as such fertilization and oocyte activation dynamics can point toward the difference, for example, between polyspermic and polygynic morphology. Male and female pronuclei, unlike in the mouse, are more similar in size, and pronuclear proximity to the polar bodies is not sufficiently predictive to allow for absolute determination of male vs. female pronuclei, but identification of pronuclear status in the human is essential [111]; correction of polyspermy has been attempted, but due to the morphological similarities between male and female pronuclei, this should remain an experimental exercise [112].

Also, exceptions to oocyte quality metrics should be monitored as an oocyte cohort, though not homogeneous, may give clues to cycle stimulation or within-patient issues. An excellent resource for photomicrographs of post-fertilization cytoplasmic structures, including pronuclei, is Papale et al. [113]. In cases where it appears that polypronuclei are present in the cytoplasm, the presence of nucleoli should be verified, as a vacuole may mimic a pronucleus in size and position relative to other nuclear structures. Early IVF studies may not have included pronuclear evaluations; rather cleavage of the ova was determined to be sufficient evidence for fertilization.

Polyspermic fertilization is common following IVF (but usually below 10% of all oocytes inseminated), and as demonstrated by Wolf et al. [88], the incidence can be directly correlated to sperm insemination concentration. There can be within-patient oocyte characteristics where a majority of the cohort are found to be polyspermic, but typically, and unless the oocytes are immature [114], the blocks to polyspermy are relatively robust. This mechanism may be overwhelmed by multiple sperm penetrations prior to membrane depolarization, or when membrane depolarization events occur slower than normal, or if cytoplasmic maturation has failed to install appropriate cortical granule populations (see [115, 116]).

Establish a baseline for poly-pronuclear fertilization; patient-to-patient and within patient variation exist, but without metrics, systemic problems with fertilization rates, including poly-pronuclear fertilization, may go unnoticed. Polyspermic and polygynic fertilization, taken together as poly-pronuclear fertilization, occur to varying proportions, and tracking these events, regardless of method of insemination, is important; egg source and fertilization method can both alter these proportions [117]. Of 7664 oocytes retrieved, 6631 oocytes were inseminated by sperm injection or conventional fertilization (20,000 sperm per 200 ul drop, up to 3 oocytes per drop). For sperm injection cycles, 0.7, 0.1, and 0.7% of oocytes were poly-pronuclear (anonymous, donor, and patient oocyte cycles, respectively), and for conventional insemination cycles, 2.2, 2.5, and 4.7% of oocytes were poly-pronuclear (anonymous, donor, and patient oocyte cycles, respectively). In another large study [118], second polar body retention – polygynic fertilization following sperm injection specifically – was estimated at 2.5% of fertilized oocytes. Data were translated to conventional fertilization cycles, where 8.1% displayed 3-pronuclei; a "true" polyspermic fertilization rate of 5.6% was calculated, essentially a twofold greater incidence of polyspermy compared to polygyny.

31.14 Post-fertilization Events

The goal of conventional fertilization is to maximize the number of monospermic fertilized ova while minimizing poly-pronuclear fertilization, without the use of micromanipulation. Post-fertilization cleavage and continued development fall under embryo culture and have been discussed in detail in many other publications (for many examples, see [119–124]).

For this chapter, it is sufficient to state that there are likely as many approaches to embryo culture and determination of viability as there are embryologists: static, dynamic, and simple media, complex media, sequential and single media, interrupted and continuous culture, and, most recently, time-lapse incubation. But detailed records should be maintained to allow for backtracking embryo developmental failures to early pre- and post-fertilization events.

31.15 When Things Go Wrong

31.15.1 Fertilization Failure

With conventional fertilization, there are fewer data points available in regard to oocyte maturation at the time of sperm-oocyte co-incubation. The cumulus cells usually mask any obvious cytoplasmic and nuclear configurations; however, detailed records of the oocyte nuclear status, at time of fertilization evaluation, will help establish a baseline for ovarian stimulation efficacy and patient variation. Each presumptive unfertilized ovum should be identified as mature (MII) or immature (MI, GV, GVBD), degenerated, broken, and so on. Data regarding oocyte maturation when using sperm injection can be used as an inference guideline for all IVF cycles. For example, nuclear maturation status (MII) for 61 anonymous donors, 20 known donors, and 288 patient oocyte sperm injection cycles yielded 85.8, 81.6, and 80.1% MII oocytes, respectively [117]. The caveat to using these data to troubleshoot fertilization failure is that for sperm injection cycles, the data are real time, while oocytes may continue to mature overnight during the co-incubation period.

Many aspects of fertilization failure can be attributed to the oocyte (for an excellent review of this topic, see [40]), the sperm, and/or a combination of the two [91, 92]. Sperm morphology, particularly in reference to acrosomal defects, a complete lack of the acrosome (globozoospermia), or other sperm pathologies, should be considered in cases of fertilization failure [90].

Sperm function tests, for example, sperm binding assays or sperm decondensation assays, might also prove useful for predicting fertilization potential [125–127] or used in follow-up to reduced or failed fertilization; but few laboratories provide real-time testing using the same sperm that was used for the insemination procedure.

Evaluation of cumulus cell dispersion, sperm motility alongside of the oocyte(s), and sperm binding to the zona are also useful. There should be evidence that sperm enzymatic release worked to break down the cumulus-hyaluronate matrix, and there should be visible evidence of sperm/zona binding with conventional insemination; even mechanical stripping of the coronal cells does not dislodge all sperm bound to the zona. If no sperm can be found bound to the zona and if delayed pronuclear formation and cleavage does not occur – and if the laboratory has the equipment – fluorescent observation may be useful as sperm may have penetrated the oocyte without activating the oocyte [105].

Rescue sperm injection may be attempted – and can be successful – provided there are sufficient safeguards in place: examination of sperm binding patterns, accurate assessment of polar body status, and observation for delayed pronuclear formation (e.g., see [128, 129]). Delayed pronuclear formation should also be considered to be a possible oocyte dysfunction, possibly related to decreased capacity by the oocyte to remodel and repair compromised sperm [130].

A note of caution regarding rescue sperm injection: sperm injection, while there is a condensed sperm cell already in the cytoplasm, may result in the activation of the oocyte and processing of both sperm; careful observation of resulting pronuclei patterns is important. Fertilization determinations are usually performed early in the work day, and as early pronuclear formation can be seen approximately 6 h following injection, it is reasonable to evaluate "rescued" oocytes on the same day as the rescue injection; reliance on cleavage alone could lead to an embryo with an abnormal chromosomal complement.

Evaluate the oocyte retrieval process: recovery and rinsing media, temperatures of surfaces and holding blocks, pipetting of the COCs, and incubation conditions (pH, bicarbonate-base medium, protein, temperature, time for dish/device pre-equilibration).

Evaluate the insemination process: was the sperm concentration dilution protocol followed, were sperm observed at the time of insemination for quantity and quality of motility, were sperm observed for motility at the time of fertilization evaluation, were cumulus cells sufficiently dispersed, and, importantly, was the protocol for sperm capacitation followed (pH, bicarbonate-base medium, protein, temperature, time)?

31.15.2 Contamination of Co-incubation Devices/Dishes/Drops

On occasion there are observations that the fertilization environment has become contaminated; bacterial and fungal contaminations are the most notable, and most often, this is discovered after the sperm-oocyte co-incubation period. Technique breakdown can contribute to this, but also consider that gamete sourcing is a contributor as well; follicular fluid and semen are not sterile body fluids. Most gamete and embryo culture media contain some form of broad-spectrum antibiotic, but at times, this is not enough to prohibit overgrowth of a contaminant. Particularly with conventional insemination, given the "sticky" nature of the COCs and the numbers of sperm used for insemination, the opportunities for contaminants to be carried through to co-incubation are greater than for sperm injection protocols where cumulus cells are stripped away, and a single sperm cell is selected. For discussions on this topic, see [19–22].

In the event of a contamination event, ova should be recovered as quickly as possible and rinsed through a series of dilutions designed to minimize carry-over. There are no strict protocols for rinsing human embryos or oocytes that have been exposed to pathogens that I am aware of; however, the International Embryo Transfer Society has developed a washing technique [131]. I am aware of two laboratories that have employed this technique successfully after a bacterial overgrowth in one case and a fungal overgrowth in the other. The technique is essentially a series of ten dilutions, moving ova in 20 ul of medium into 2 ml, repeating this process with a new dish of medium and a new pipette tip each time; smaller volumes can be used, but the protocol stresses the importance of maintaining the 1:100 dilution factor for each of the ten steps.

31.16 Summary

It is increasingly important for each laboratory to maintain at least one functional protocol for conventional insemination; not all programs have joined the movement toward performing sperm injection on all cycles, as is required for increased use of genetic testing of embryos. And in some laboratories, the desire to ease the burden of managing personnel and resources may lead all cycles utilizing sperm injection. Regulations and opinions (providers and patients, as to the safety of sperm injection) vary; but also consider the importance of maintaining conventional insemination techniques in the event that the sole micromanipulation system in the lab breaks down or the sperm injection specialist is indisposed. Understanding the details is critical to the process of quality management; things will go wrong, and finding a solution, as quick as possible, is the responsibility of all members of the laboratory staff. Last, historically, it is good for all of us to remember the origins of IVF, the critical moments where the industry as we know it today was formed.

Review Questions

1. List the in vivo physiological conditions regulating sperm capacitation that can be translated to the in vitro process.
2. List the visible physiological change(s) that occur during sperm capacitation.
3. List three physiological failure points for troubleshooting a cycle with completely failed fertilization.
4. Identify the physiological and nonphysiological functions performed by protein supplements.
5. Describe the differences between polyspermic and polygynic pronuclei and identifiers of each condition, and detail an approach to remedy each condition.

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Intracytoplasmic Sperm Injection

Gianpiero D. Palermo, Nigel Pereira, Queenie V. Neri, Stephen Chow, Stephanie Cheung, and Zev Rosenwaks

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Learning Objective

- To provide an overview of the reason that commenced the development of intracytoplasmic sperm injection and the current indications and address the safety concerns regarding the procedure

32.1 Introduction

ICSI involves the insertion of a single spermatozoon into the oocyte, bypassing all the egg coat penetration and gamete fusion steps characteristic of natural fertilization. This was first achieved in sea urchin [49], in mouse [62], and later in hamster eggs [134]. This micromanipulation approach was also plagued by oocyte injury and lysis [73], with only about 30% of injected mouse eggs surviving the procedure [131]. Because the sperm-egg fusion step is bypassed in ICSI, male pronucleus development generally required oocyte activation in most species tested. This was achieved by the vigorous suction of ooplasm prior to sperm nucleus insertion [98] or by exposure to A23187 [50, 143].

The first ICSI offspring were obtained in the rabbit following the transfer of sperm-injected eggs into the oviduct of a pseudopregnant female [51], with a bovine live birth reported soon thereafter [44]. Although applied to human gametes some years earlier [59], the first human pregnancies from ICSI were established only in 1992 [85], and since then, hundreds of thousands of ICSI babies have been born [85, 92, 99]. ICSI has made conception and parenthood possible for couples with many forms of male factor infertility and at rates similar to those in patients treated by the standard IVF with apparently normal gametes [80, 88].

32.1.1 Indications

Despite agreement in some areas, no universal standards for patient selection have been defined. However, there is a general consensus that ICSI be adopted when an extremely poor sperm sample is noted or following fertilization failure using in vitro techniques. Thus, in addition to male factor infertility, ICSI may be utilized in non-male factor settings as well.

When initial sperm concentration in the ejaculate is $<5 \times 10^6$/ml, the likelihood of fertilization with standard IVF is significantly reduced [144], and therefore, such couples should be considered unsuitable for this procedure, particularly where <1% normal forms are observed. However, fertilization of mature oocytes may still fail to occur in the presence of normal sperm [15] because of a hardening of the zona pellucida [22], or when oocytes reveal ooplasmic inclusions [2, 136]. Abnormalities of the zona pellucida prevent sperm fusion with the oolemma [57] thus justifying sperm injection. In most instances, however, failure of fertilization is due to coexisting sperm abnormalities presenting ICSI as the only treatment option [89].

Early experience showed that isolated nuclei of testicular and epididymal hamster spermatozoa decondensed soon after injection into mature hamster oocytes and formed pronuclei in activated eggs [135]. Although in vitro fertilization of human oocytes was accomplished in men with epididymal spermatozoa recovered in cases of obstructive azoospermia [117, 129], only with the advent of ICSI was it possible to obtain consistent fertilization with them [88, 118, 132]. Testicular biopsy was employed to obtain sperm cells from men who had a scarred epididymis and therefore, no chance of retrieval through that route [19, 36, 114]. However, the therapeutic possibilities of ICSI go even further since immotile testicular spermatozoa and even spermatids have been successfully used [130].

Some men produce only round-headed spermatozoa which have no acrosome and can neither bind to nor penetrate zona-free hamster oocytes [58, 70]. However, ICSI has enabled even such acrosomeless spermatozoa to establish pregnancies [32, 47, 64, 127, 128]. Moreover, ICSI's dependability has broadened its initial use from a technique capable of overriding the dysfunctionality of spermatozoa to one that may partly compensate for problems with the egg. Indeed, ICSI has allowed successful fertilization when only a few and/or abnormal oocytes were available [67]. Stripping cumulus cells from the oocytes allows a direct assessment of maturation, thus offering a woman with a limited number of oocytes a much greater chance of successful fertilization. In fact, the availability of ICSI has been instrumental in some European countries that include Italy and Germany in circumventing restrictive legislation that limits the number of oocytes inseminated or embryos to be replaced [6, 68, 69].

ICSI has also made possible a more consistent fertilization of cryopreserved oocytes [100] – overcoming the problem that freezing can lead to a premature exocytosis of cortical granules, resulting in zona hardening and inhibition of natural sperm penetration [52, 107, 120, 136]. ICSI is also the preferred conception method during the application of preimplantation genetic diagnosis (PGD). It avoids DNA contamination from additional sperm adhering to the zona, and it enhances the number of fertilizable oocytes and so embryos available for screening [34].

ICSI also has an impact in the arena of HIV infection. Three quarters of individuals infected by HIV or HCV are in their reproductive years. Male-to-female transmission of HIV is estimated to be only 1 per 1000 acts of unprotected intercourse [25] and even fewer in HCV-infected patients [39]. Moreover, because of antiretroviral therapies, the course of HIV-1 infection has shifted from a lethal acquired immunodeficiency syndrome to a chronic manageable disease. Though many patients infected with HIV-1 show interests in beginning a family, most serodiscordant couples are concerned, nevertheless, with the possibility of both horizontal and vertical transmissions of the virus. In such cases, intrauterine insemination (IUI) with spermatozoa processed by double gradient centrifugation followed by the swim-up has been the preferred method of treating serodis-

cordant couples with an HIV-1-infected male partner [14]. However, the use of ICSI has been proposed by several groups because of its negligible semen exposure, thereby reducing the risk of viral transmission [74, 105]. Advantages of ICSI over IUI also include the considerably higher success rate [74], requiring fewer attempts to achieve pregnancy while reducing viral exposure [96]. Fortunately, so far, no seroconversions have been reported following ART treatments including IUIs [137].

Finally, because only a single spermatozoon is needed for each egg, ICSI has allowed treatment of men who are virtually azoospermic (also defined as cryptozoospermic) [7]. Such cases of spermatogenic arrest have necessarily involved the injection of immature spermatozoa or even spermatogonia [31, 36, 130, 133]. Nonetheless, where fertilization occurs in such cases, conception is accomplished with embryo implantation following a similar pattern, at least in our experience, to that seen in IVF.

32.2 Equipment

- Micromanipulation system (NAI-2P, Narishige International USA, Inc.)
  - Hydraulic microinjector (IM-6), modified with a metal syringe (SYR-15)
  - Injector (IM-9C), air-filled
  - BDH oil – for loading the injector (BDH Laboratory Supplies, Poole, Dorset, England)
- Inverted microscope with Nikon Polarized Optics CFI S Plan Fluor (20× and 40× objectives) and CFI Apo (2×, 4×, and 10× objectives) (TE2000U, Nikon USA, Melville, New York, USA)
- Vibration-free table (Newport Research Corporation, Irvine, California, USA)
- Custom-designed horseshoe-shaped heated stage (Easteach Laboratory, Maspeth, New York, USA)
- Microtools (Vitrolife, San Diego, California, USA)
  - Injection pipette (15415; 4–5.5 μ ID)
  - Injection pipette (15346; 5–7 μ ID)
  - Holding pipette (15305)
- Oocyte transfer and denudation pipettes (hand-pulled, flame-polished Pasteur pipettes)
- ICSI dish (BD Falcon 351006; Becton, Dickinson & Co., Franklin Lakes, New Jersey, USA)
  - Stereomicroscope (SZX12, Olympus America Inc., Center Valley, Pennsylvania, USA)
  - Distriman® Repetitive Pipette (F164001, Gilson, Inc., Middleton, Wisconsin, USA) – ICSI medium dispenser
  - AutoRep™E Electronic Repeating Pipette (AR-E1, Rainin Instrument LLC, Oakland, California, USA) – oil dispenser
  - Pipetman P20 (F123600, Gilson, Inc.) – PVP and sperm loading

32.2.1 Reagents

- ICSI Cumulase® (16125000, MediCult, Origio, Mt. Laurel, New Jersey, USA)
- Embryo culture medium (home-brew, modified Cornell medium based on G1™ and G2™ components) [37, 38]
- ICSI injection medium (G-MOPS™ [10129] supplemented with 6% G-MM™ [10038], Vitrolife, Inc., Englewood, Colorado, USA)
- Tissue culture oil (ART-4008, SAGE In-Vitro Fertilization, Inc. A CooperSurgical Company, Trumbull, Connecticut, USA)
- 7% PVP with HSA (90121, Irvine Scientific, Santa Ana, California, USA)

32.3 Sperm Preparation

32.3.1 Ejaculated Sample

Semen samples are collected by masturbation after at least 2–3 days of abstinence; they are then allowed to liquefy for about 20 min at 37 °C prior to analysis. Other methods of semen collection such as electroejaculation and retrograde ejaculation have been described elsewhere [88].

Semen concentration and motility are assessed in a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel). As might be expected, morphologic quality of the sperm has a significant positive correlation with male infertility. This evaluation is performed using the strict criteria of Kruger et al. [56]. Evaluations are carried out by spreading 10 μl of semen or sperm suspension on prestained slides (Cell-Vu®, Pre-stained Morphology Slides [DRM-900], Fertility Technology, Marietta, Georgia, USA), which can provide rapid results. At least 200 spermatozoa/slide are categorized microscopically at 100× under oil immersion. Two counts are performed for both concentration and morphology. Semen quality is considered suboptimal when the sperm concentration is $<20 \times 10^6$/ml, the progressive motility is <40%, or the proportion of spermatozoa with normal morphology is <4% of the spermatozoa.

For selection of spermatozoa, the sample is washed by centrifugation at 600 g for 5 min in HTF medium supplemented with 0.4% HSA. Semen samples with $<5 \times 10^6$/ml spermatozoa or <20% motile spermatozoa are washed in a home-brew medium by a single centrifugation at 600 g for 5 min. The resuspended pellet is layered on a discontinuous density gradient (Isolate®, Irvine Scientific) on two (90% and 50%) or single (90%) layers and then centrifuged at 300 g for 20 min when samples have a sperm density of $<5 \times 10^6$/ml spermatozoa and <20% motile spermatozoa. The sperm-rich fraction is rinsed by adding 4 ml of culture medium and centrifuged at 500–1800 g for 5 min to remove silica gel particles. For spermatozoa with poor kinetic characteristics, the sperm

suspension is exposed to a 3 μM solution of pentoxifylline for 15 min and centrifuged one more time. The concentration of the assessed sperm suspension is adjusted to 1–1.5 × 10^6/ml, when necessary, by the addition of HTF medium, and subsequently incubated at 37 °C in 5% CO_2 in air.

32.3.2 Surgically Retrieved Spermatozoa

Azoospermia can be indicative of epididymal vas obstruction or absence (obstructive azoospermia) or a failure of spermatogenesis (non-obstructive azoospermia). Obstructive azoospermia is characterized by normal sperm production and is often caused by congenital bilateral absence of the vas deferens, often associated with a cystic fibrosis gene mutation(s). The treatment for these men is microsurgical epididymal sperm aspiration (MESA), or when the epididymal access is lacking, direct testicular sampling is undertaken. On the other hand, non-obstructive azoospermia is characterized by a varying degree of spermatogenetic failure and may be associated with certain chromosomal abnormalities [41, 101]. The only method to retrieve spermatozoa from this form of azoospermia is a direct extraction of spermatozoa from the testis.

32.3.3 MESA

The infertility of men due to irreparable obstructive azoospermia has been treated successfully by microsurgical epididymal sperm aspiration (MESA) [111, 112] or percutaneous testicular retrieval of spermatozoa [115]. In the MESA procedure, 1–5 μl of fluid is aspirated from the lumen of an individual epididymal tubule in the midportion of the obstructed epididymis with a 300–350 μm glass pipette. The epididymal fluid is diluted with 300 μl of culture medium. Additional proximal punctures of the epididymis are performed until enough spermatozoa are obtained. Because sperm concentration in the epididymal fluid often reaches over 1 × 10^6/μl, only microliter quantities generally suffice. Epididymal fluid is diluted in 500 μl culture medium and processed like ejaculated spermatozoa. After removal of the density gradient medium, a 1 μl aliquot of the final suspension at approximately 1 × 10^6/ml is placed in the injection dish [86, 88].

32.3.4 TESE

In the case of men with non-obstructive azoospermia, spermatozoa are retrieved directly from the testis. Typically, multiple biopsies are needed to find the rare spermatogenetic foci that are present within the testes of such men. Biopsies are performed under optical magnification to identify and preserve the sub-tunical testicular blood supply with biopsy incisions made in avascular regions [113].

Each biopsy specimen is first rinsed in culture medium to remove red blood cells, separated into individual tubules on sterile glass slides, and minced using fine scissors. The resulting suspension of seminiferous tubules is then sequentially passed through a 24 gauge angiocatheter to further disrupt the tubules. Individual testicular samples are distributed in 5.0 ml centrifuge tubes (Falcon, Becton Dickinson and Company, Lincoln Park, NJ, USA) containing an excess of culture medium. To assess for the presence of spermatozoa, a small amount (~5 μl) of suspension medium is carefully studied under a phase-contrast microscope at 200–400×. In preparation for ICSI, the shredded testicular tissue is removed and the suspension medium subsequently centrifuged at 500–1800 g for 5 min, with the pellet being subjected to a single-layer density gradient centrifugation. It has been possible to identify individual and enlarged seminiferous tubules that presumably are supporting spermatogenesis in contrast to the surrounding thin sclerotic tubules. Biopsy samples are placed into 200–300 μl aliquots of culture medium on a glass slide and examined under a phase-contrast microscope and repeated until the presence of spermatozoa is documented. The specimen is then placed directly into a 40 μl drop under oil where the tips of two 25 gauge needles on 1 ml syringes are used to shred the testicular tissue. Subsequently, the tissue is removed from the droplet, and 3 μl of the suspension is placed directly onto the injection dish.

32.3.5 Micro-TESE

In cases of non-obstructive azoospermia, the procedure for direct microscopic identification of functioning seminiferous tubules is referred to as microdissection TESE [110]. As with the standard multi-biopsy approach, optical magnification (6–8 power) is used to visualize blood vessels under the surface of the tunica vaginalis, allowing testis biopsy incisions to be made in avascular regions. Instead of planning for multiple incisions in the tunica albuginea, an attempt is made to open it widely by creating the incision on the testis' midportion. This optimizes exposure of the testicular parenchyma without disrupting its blood supply. Direct examination is carried out at 20–25× under the operating microscope, during which an attempt is made to identify individual seminiferous tubules that were larger than others in the testicular parenchyma. Small (2–10 mg) segments are excised sharply from tubules that are larger and more opaque (whiter). Each excised specimen is further cut into smaller pieces to promote the release of any spermatozoa, with the resulting suspension examined as in standard TESE. Although additional incisions in the same or contralateral testes can be made until spermatozoa are retrieved or when further biopsies would appear to compromise the blood supply of the testes. Once spermatozoa are found, the procedure is terminated and testis sutured.

When no spermatozoa are identified, testicular tissue is placed in 1 ml of pre-warmed medium supplemented with 5% HSA, 1.6 mM $CaCl_2$ (Sigma Chemical Co., St Louis, MO), 25 μg/ml DNase (Sigma Chemical Co.), and 1000 IU/ml

collagenase type IV (Sigma Chemical Co.) [102]. DNase is added to the incubation medium to prevent clotting of the resulting cell suspension due to the release of free DNA from apoptotic cells [17, 18]. Testicular tissue is exposed to collagenase and incubated at 37 °C for 1 h. The suspension is pipetted every 10–15 min to enhance enzymatic digestion. Large portions of tubular walls are removed with fine tweezers. The digested suspension is then centrifuged twice at 500 g for 5 min. The supernatant is removed and the pellet re-suspended in 500 µl of sperm cell medium.

32.4 Oocyte Collection

Superovulation is performed by administration of gonadotropins in association with agonist or antagonist protocol [27]. Human chorionic gonadotropin (hCG) is administered when criteria for oocyte maturity are met, and oocyte retrieval by vaginal ultrasound-guided puncture is performed 35 h later. Under the inverted microscope at 100×, the cumulus corona cell complexes are scored as mature, slightly immature, completely immature, or slightly overmature. Thereafter, the oocytes are incubated for more than 4 h. Immediately prior to micromanipulation, the cumulus corona cells are removed by exposure to HTF-HEPES-buffered medium containing 40 IU/ml of ICSI Cumulase®. The removal is necessary for observation of the oocyte and effective use of the holding and/or injecting pipette during micromanipulation. For final removal of the residual corona cells, the oocytes are repeatedly aspirated in and out of a hand-drawn Pasteur pipette with an inner diameter of ~200 µm. Each oocyte is then examined under the microscope to assess the maturation stage and its integrity, metaphase II (MII) being assessed according to the absence of the germinal vesicle and the presence of an extruded polar body. ICSI is performed only in oocytes that have reached this level of maturity.

32.5 Intracytoplasmic Sperm Injection

32.5.1 Loading Tools

The holding and injection pipettes are inserted into the respective micromanipulation tool holders mounted on an inverted microscope. The controllers are pneumatic for the holding pipette and oil-filled for the injection pipette. Using the coarse motorized controllers, the pipettes are positioned in the center of the microscopic field at 20×, and then the magnification is gradually increased while maintaining the tools in focus by adjusting the hydraulic controllers. Under the highest magnification (400×), correct pipette positioning is achieved only by the use of the hydraulic joysticks, and both pipettes should be able to course through the entire optical field. With regard to tool tip angles, the distal bent portions of both microtools should be slightly above parallel to avoid the elbows touching the bottom of the dish and interfering with the control. This also allows prompt immobilization and visual control of the spermatozoon inside the injection pipette. Once properly aligned, the pipettes are raised by means of the coarse motorized controllers to allow placement of the ICSI dish on the microscope stage.

32.5.2 ICSI Dish Preparation

Nine drops containing 8 µl of injection medium are placed in a petri dish, with one in the center radially surrounded by the other eight (Fig. 32.1). The drops should be as close together as possible to allow full visualization within the 20 mm opening on the heated stage. The drops should then be gently overlaid with culture oil to prevent evaporation. Using a red non-embryo toxic wax pencil, the 12 o'clock position is marked, a circle is drawn around the central drop, and the drops are sequentially numbered starting from the 12 o'clock position, moving counterclockwise. This allows easy navigation between droplets during ICSI. ICSI dishes are stored at 37 °C until use.

32.5.3 Loading Gametes into the ICSI Dish

Immediately prior to the injection under a stereomicroscope, the central drop is removed and replaced with 1 µl of sperm suspension diluted in 4 µl of 7% PVP. Using a hand-pulled Pasteur pipette, MII oocytes are aspirated from the culture dish, and a single oocyte is placed in each drop.

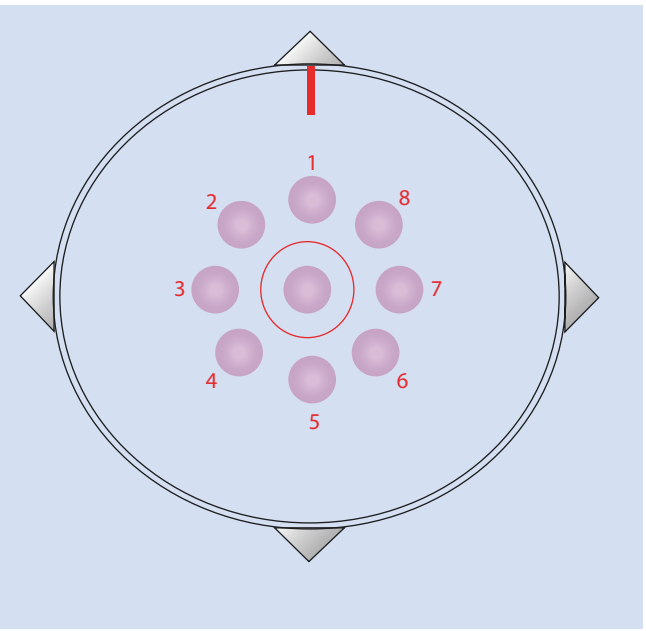

Fig. 32.1 The drawing depicts a petri dish containing the droplets of medium (8 µl) and sequentially numbered going counterclockwise. The center droplet is removed and replaced with PVP and sperm, while in each surrounding droplet, an individual oocyte will be placed

32.5.4 Sperm Immobilization

Position the spermatozoon at 90° with respect to the pipettes' tip, and gently lower the cylindrical tip to compress the principal piece of the tail, by rolling the posterior flagellum over the bottom of the petri dish (Fig. 32.2a–c). If initially unsuccessful, the procedure is repeated until the tail is clearly kinked, looped, or convoluted (Fig. 32.2d, 1–3). It is important to note, however, that a misshapen tail may adhere to the dish or to the inner surface of the pipette. The spermatozoon is aspirated tail first. The injection needle is lifted slightly via the two control knobs of the joystick to avoid damaging the needle spike. The microscope stage is then repositioned until the injection needle enters the oocyte drop. It is important to note that the difference in media consistency (PVP versus culture medium) may allow the sperm to move distally into the pipette and become loose.

32.5.5 Injection

To find the egg, the magnification is briefly lowered to 200×, and once the egg is centrally in the field, the magnification brought back to 400×. The oocyte is held in place by suction through the holding pipette, and, using both tools, the oocyte is rotated slowly to locate the polar body and the area of cortical rarefraction (or polar granularity) (Fig. 32.3a). When the equatorial plane of the oocyte is located, the depth of the holding pipette is adjusted to have its internal opening in the same plane. This allows for greater support of the holding pipette in a position opposite to the injection point. It is ideal to have the inferior pole of the oocyte touching the bottom of the dish, as it affords a better grip of the egg during the injection procedure. The injection pipette is lowered and focused with the outer right border of the oolemma on the equatorial plane at 3 o'clock. Bring the spermatozoon close to the beveled opening of the injection pipette, then bring the pipette to the zona, press against it to begin penetration, and thrust forward to the inner surface of the oolemma at 9 o'clock (Fig. 32.3b–d). At this point, a break in the membrane should occur at the approximate center of the egg (Fig. 32.3e). Such a break is indicated by a sudden quivering of the convexities of the oolemma (at the site of invagination) above and below the penetration point (Fig. 32.3f), as well as by the proximal flow of cytoplasmic organelles and the spermatozoon back into the pipette. The spermatozoon is then ejected with the cytoplas-

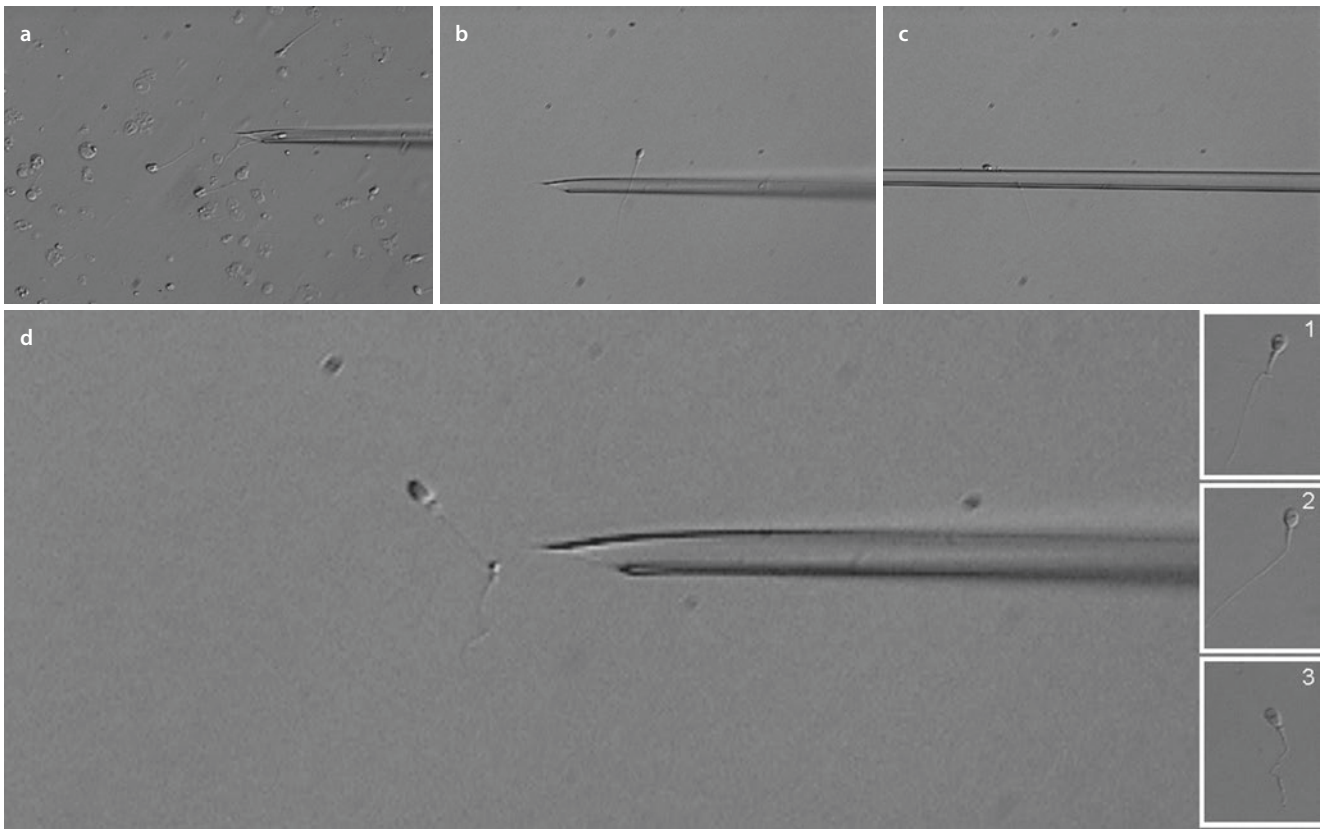

Fig. 32.2 Aggressive immobilization of a testicular spermatozoon prior to intracytoplasmic sperm injection. **a** A single spermatozoon displaying a satisfactory morphology is aspirated from a sperm droplet. Such droplets are made without PVP in cases where sperm count and motility are poor. **b** The chosen spermatozoon is then moved into the PVP droplet, where the injection pipette is gently lowered onto the sperm tail main piece, compressing it. Even though the spermatozoon is immotile, immobilization is still carried out to ensure the best chance of fertilization, since a damaged sperm membrane facilitates exposure of the nucleus to the ooplasm. **c** While maintaining downward pressure on the flagellum, the injection pipette is moved back and forth, effectively rolling the spermatozoon on the dish bottom. **d** Once immobilization is completed, the tail should be permanently distorted – either looped (1), kinked (2), or convoluted (3)

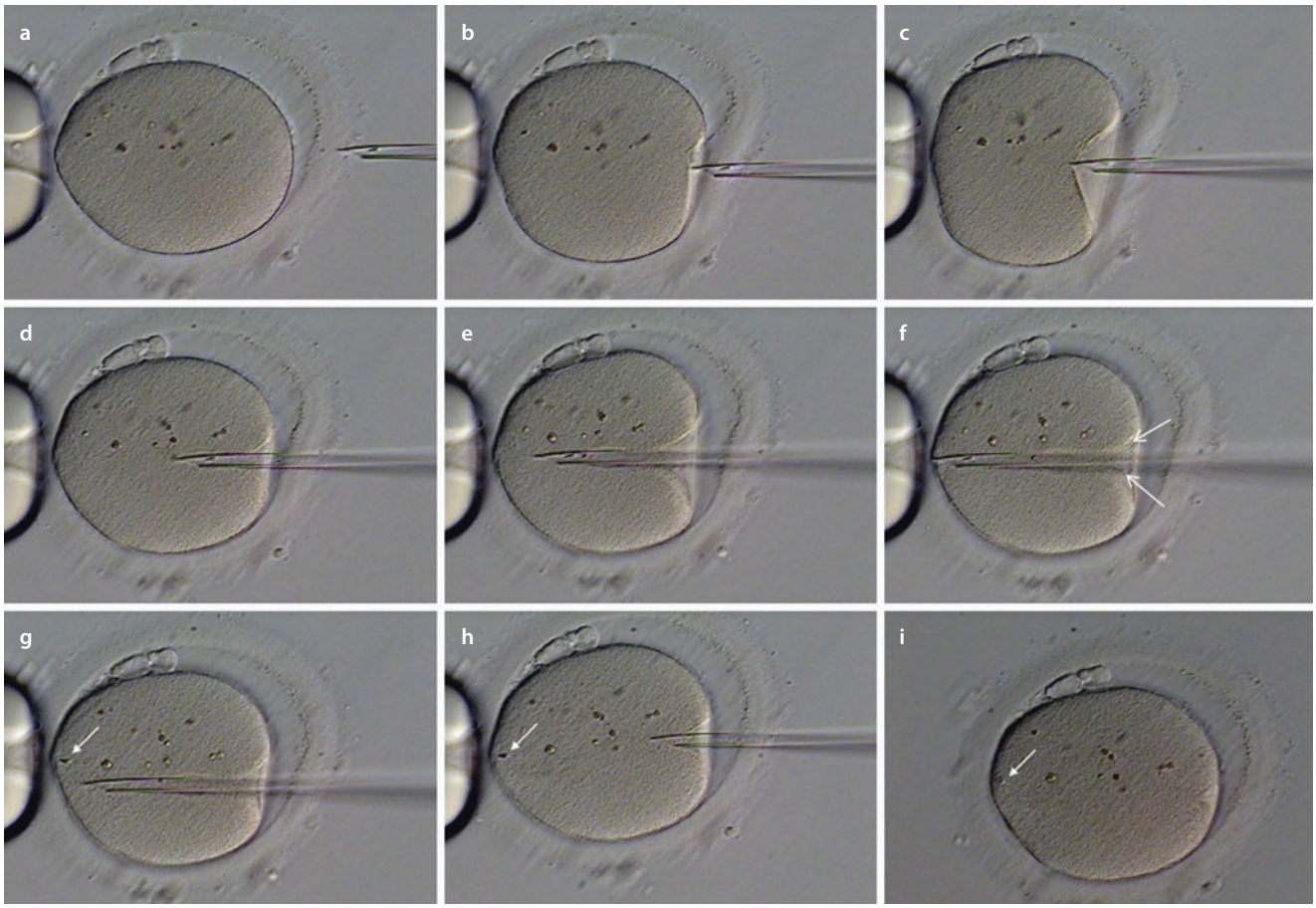

Fig. 32.3 Intracytoplasmic sperm injection of a human oocyte. **a** The oolemma at the 3 o'clock portion of the oocyte is brought into focus, as are the holding (left) and injection pipette (right) tips. Suction is applied on the holding pipette to stabilize the oocyte, and the spermatozoon is advanced to the tip of the injection pipette. **b** Advancement of the injection pipette through the zona at the oocyte's equatorial plane bypasses the zona and brings the pipette spike into contact with the oocyte membrane. **c–e** Its elasticity creates a funnel around the injection pipette as it moves deeper into the oocyte. **f** Once the oolemma is penetrated, there is a distinct quivering of the oocyte membrane, which then returns to its original position (arrows). **g** As the sperm is deposited in the ooplasm (arrow), a small amount of cytoplasm is aggressively aspirated and released to facilitate oocyte activation. **h** Removal of the injection pipette is accompanied by gentle aspiration from the center of the oocyte outward to facilitate resealing of the membrane by bringing the borders of the injection funnel into closer proximity. **i** An injected oocyte with sperm in the cytoplasm (arrow), displaying minimal evidence of tool penetration at its 9 o'clock edge

mic component (Fig. 32.3g). To optimize its interaction with the cytoplasm, the sperm should be ejected past the tip of the pipette to ensure a close intermingling with the ooplasmic lattices, which help maintain the sperm in place while withdrawing the pipette (Fig. 32.3h). To induce oocyte activation, additional ooplasm is aspirated back and forth with the injection pipette. It is paramount to avoid leaving behind residual medium with the spermatozoon as well as closing the breach of penetration. This is accomplished by generating a mild suction while removing the pipette. To do this, when the pipette is at the approximate center of the egg, some surplus medium is reaspirated so that the cytoplasmic structures can envelop the sperm, thereby reducing the size of the breach (Fig. 32.3i). This also expedites the closure of the terminal part of the funnel-shaped opening at 3 o'clock. Once the pipette is extracted, the edges of the entry point should maintain a funnel shape with the tip toward the center of the egg. If the border of the oolemma becomes everted, the cytoplasmic organelles can leak out, and the oocyte may lyse. The average time required to accomplish a single oocyte injection is about 30–40 s.

32.6 Embryo Development and Clinical Outcome

The popularity of ICSI to overcome almost all forms of male infertility has increased remarkably since its inception [93]. For example, the use of ICSI has increased from 36.4% in 1996 to 76.2% in 2012 in the USA [12]. Furthermore, the International Committee for Monitoring Assisted Reproductive Technologies reported that the global utilization of ICSI was 66% based on data from 2500 ART clinics in 58–61 countries between 2008 and 2010. However, large disparities were reported in ICSI utilization, i.e., 55% in Asia, 65% in Europe, and 100% in the Middle East [28].

There has also been a steady and progressive increase in ICSI utilization at our center starting at 32.2% in 1993 rising to 48.8% in 1995 and reaching 74% in the mid-2000s. In the last 23 years at Cornell, we have performed a total of 39,918 ART cycles with an average maternal age for IVF of 36.6 ± 4 years and for ICSI of 37.3 ± 5 years and mean paternal ages of 39.5 ± 7 years and 39.9 ± 8 years, respectively. Of those cycles, 33.2% (13,253) included the standard in vitro insemination of 138,815 oocytes with a fertilization rate of 63.6% and a clinical pregnancy rate of 41.5%, the remainder being ICSI cycles. IVF was generally performed in patients with normal semen, while ICSI has been used to treat couples with doubtful spermatozoa, a history of poor fertilization, and/or limited numbers of oocytes. Nonetheless, the two systems consistently generated similar results.

Of 18,863 couples treated by ICSI, the average number of oocytes retrieved was 10.56 per cycle (n = 349,560), of which 279,361 (80.0%) were at metaphase II and, therefore, subjected to ICSI. Of the oocytes injected, 94.93% (265,189/279,361) survived and 206,110 (73.78%) developed two pronuclei (PN). Of the oocytes that were abnormally fertilized, 7315 (2.62%) displayed 1 PN and 9709 (3.48%) 3 PN.

The types of mature spermatozoa included 26,397 ejaculated samples, 3684 cryopreserved, 89 obtained by electroejaculation, and 53 by bladder catheterization (Table 32.1). Among the collection/cryopreservation methods, fertilization rates ranged from 68.6% to 77.2%. The clinical pregnancy rate was comparable in all groups.

When more immature forms of spermatozoa were utilized, for example, those surgically retrieved, the fertilization rate of 58.6% was satisfactory but lower than that with ejaculated spermatozoa ($P < 0.0001$) (Table 32.2). While the clinical pregnancy rate was lower in the ejaculated group in comparison to the surgically retrieved spermatozoa, this difference may be attributed to the maternal age. The etiology of the *vas deferens or efferens* obstruction, congenital or acquired, had no effect on fertilization after ICSI giving an overall rate of 72.2% and a clinical pregnancy rate of 52.1%. However, after cryopreservation of epididymal spermatozoa, the clinical pregnancy was dramatically reduced from 60.9% to 47.6% ($P < 0.0001$).

In testicular extraction cases, with the compromised spermatogenesis typical of the non-obstructed azoospermic patient, the spermatozoa recovered generated a fertilization rate of 51.2% and a clinical pregnancy rate of 37.7%. When comparing fresh versus cryopreserved spermatozoa in testicular cycles, the clinical pregnancy rate was comparable between the two groups (40.5% versus 31.8%).

Of 33,111 ICSI cycles analyzed, 14,995 resulted in a positive ßhCG (51.9%), and in about 39.3% of all cycles, the presence of at least one fetal heartbeat was observed (Table 32.3). Among the positive ßhCG, 2619 (17.5%) were biochemical pregnancies, 855 (5.7%) were anembryonic, and 178 (1.2%) were ectopic. Among the 11,343 cycles with a fetal heart, 1320 spontaneously miscarried. This left an ongoing pregnancy rate of 30.3% per retrieval (10,023/33,111) and 34.7% per embryo replacement procedure (10,023/28,894).

When 30,796 ICSI cycles (after exclusion of the donor egg cycles) were plotted as a function of increasing maternal age, there was a progressive decrease in pregnancy ($P < 0.0001$) (Table 32.4) and consequently delivery rates ($P < 0.0001$). As predicted, there was a higher incidence of miscarriages, therapeutic abortions, and overall pregnancy losses as a function of the age of the female partner ($P < 0.0001$), pregnancy wastage being 2.4 times greater in women ≥40 years compared to those of <35 years.

A total of 9445 ICSI patients delivered 12,562 babies comprising 6241 males and 6143 females (with 178 unknown genders). A total of 3.3% (268) exhibited congenital abnormalities at birth, of which 130 were major and 138 were minor. IVF children (n = 4999) had a 3.1% overall malformation rate

Table 32.1 ICSI outcome according to semen source

Semen origin	Cycles	Fertilization (%)	Clinical pregnancies (%)
Fresh ejaculate	26,397	164,115/217,640 (75.4)	8950 (33.9)
Frozen ejaculate	3684	23,875/31,237 (76.4)	1132 (30.7)
Fresh electroejaculate	70	546/711 (76.8)	35 (50.0)
Frozen electroejaculate	19	140/204 (68.6)	6 (31.6)
Retrograde ejaculate	53	363/470 (77.2)	20 (37.7)

Table 32.2 Fertilization and pregnancy rates according to the origin of spermatozoa

No. of	Ejaculated	Surgically retrieved
Cycles	30,223	2888
Maternal age (M years ± SD)	37.7 ± 5 (a)	34.3 ± 5 (a)
Fertilization (%)	189,039/250,262 (75.5) (b)	17,071/29,099 (58.6) (b)
Clinical pregnancies (%)	10,143 (33.6) (c)	1200 (41.6) (c)

(a) Student's t-test, two independent samples; difference in maternal age, $P < 0.0001$
(b) χ^2, 2 × 2, 1 df, effect of sperm source on fertilization rate, $P < 0.0001$
(c) χ^2, 2 × 2, 1 df, effect of sperm source on clinical pregnancy rate, $P < 0.0001$

Table 32.3 Pregnancy characteristics of 33,111 ICSI cycles

	No. of (%)	Negative outcomes	
ICSI cycles	33,111		
Embryo replacements	28,894		
Postive bHCGs	14,995 (45.3)		
		Biochemical pregnancies	2619
		Blighted ova	855
		Ectopic pregnancies	178
Patients with fetal heartbeats	11,343 (34.3)		
		Miscarriages/ therapeutic abortions	1320
Deliveries and ongoing pregnancies	10,023		

Table 32.4 The relationship of maternal age to ICSI outcome

	Maternal age		
No. of cycles with (%)	<35	35–39	≥40
Cycles	9126	10,864	10,806
(a) Embryo replacement	8086	9528	9170
(b) Clinical pregnancy (+FHB) (% on a)	4170 (51.6) (d)	3926 (41.2) (d)	2082 (22.7) (d)
(c) Delivery and ongoing pregnancy (% on a)	3901 (48.2) (e)	3498 (36.7) (e)	1570 (17.1) (e)

(d) (e) χ^2, 3 × 2, 2 *df*, effect of maternal age on pregnancy outcome, $P < 0.0001$

(90 major and 65 minor). Major malformations ranged from cardiac defects to multiorgan diseases including central nervous system anomalies, chromosomal abnormalities (gonosomal trisomies such as 47, XXX and 47, XXY and autosomal trisomies such as chromosomes 7, 18, 21), and urogenital disorders requiring surgery such as severe hypospadias and undescended testes. Examples of minor malformations were café au lait spots, urethral defects, and very mild form of clubfoot.

32.7 ICSI Failure

ICSI failure can occur on three different levels: a failure to obtain sperm for injection, failure of fertilization after injection, and a failure to establish a pregnancy after embryo transfer.

Failure to Obtain Sperm: In men with non-obstructive azoospermia, rarely it is impossible to retrieve any spermatozoa from the testis. On the other hand, as might be expected, sperm are recovered in virtually all attempts involving men with obstructive azoospermia.

Failure of Fertilization After ICSI: As noted above, fertilization failure occurs in about 2% of ICSI cycles, whether male infertility is present or not [8, 64]. The reasons for these few failures remain unclear but probably involve either poor sperm viability or an asynchronous oocyte maturation [33].

Some men carrying acrosomeless (also known as globozoospermic) spermatozoa cannot achieve syngamy even after ICSI [1, 64, 104]. However, successful fertilization, pregnancy, and offspring have resulted after treating the oocytes post-ICSI by either a chemical agent [47, 104, 140, 141] or an electrical pulse [72, 140]. Similarly, the chances of fertilization in these problematic cases may sometimes be enhanced by utilizing sperm-derived activating extracts or calcium-releasing compounds [77, 91]. Oocyte activation agents include calcium ionophore [50], electrostimulation [140], and strontium [141]. Such adjunct treatments help to activate the oocyte by increasing the Ca^{2+} permeability of the cell membrane, thereby allowing extracellular Ca^{2+} flow into the oocyte and also inducing Ca^{2+} release from the intracellular calcium stores. In one of the first series of assisted oocyte activations, in patients with previous fertilization failure [47], the authors first injected a spermatozoon together with $CaCl_2$ and, thereafter, a calcium ionophore – this dual exposure resulted in an overall fertilization of >70%. These approaches have been able to trigger oocyte activation and allow concurrent sperm nuclear decondensation and, therefore, zygote development. To further enhance oocyte activation and syngamy, spermatozoa may be exposed to an agent that facilitates sperm membrane permeabilization [42, 53].

The inability to activate an egg has been attributed to the absence of a specific sperm protein that has been investigated in many occasions [91, 95, 139]. More recently, however, one such factor – phospholipase C-ζ1 (PLCζ1) – has been identified in mice. Following its injection into mouse oocytes, PLCζ1 was capable of inducing calcium oscillations that were identical to those observed following fertilization by a sperm [106, 126]. As an interesting correlate, the PLCζ1 isoform was undetectable in sperm of men that experienced fertilization failure with ICSI [142]. Taken together, these findings open a new avenue in the diagnosis and potential treatment of couples whose ICSI cycles terminate abruptly with unexpectedly low or definitive absence of fertilization.

Failure to Conceive After ICSI Fertilization: It must be emphasized finally that the implications of one fertilization failure for subsequent ICSI attempts are not necessarily bleak. When couples with failed ICSI fertilization attempted further ICSI cycles, their success rates were similar to other couples undergoing repeat ICSI cycles [32, 47, 48, 76].

On the other hand, as with IVF, some couples fail to conceive despite repeated ICSI cycles where the causes range from centrosomal dysfunction to DNA abnormalities [35, 87, 91]. Two hundred couples who had 433 cycles of ICSI treatment without success subsequently had 23 (12%) live births with no further treatment within 4 years of the last ICSI cycle [84]. The only predictor of live birth after ICSI failure was a shorter time to pregnancy (the time between seeking infertility treatment and the actual achievement of a pregnancy), suggesting that unknown factors contributing to longer duration of infertility were barriers to success [65].

32.8 Safety

Notwithstanding the large number of babies born from the ICSI procedure worldwide, concerns still exist as to whether the use of suboptimal spermatozoa can result in genomic abnormalities in the progeny [66]. These qualms are not only limited to the inheritance of specific traits that bear on fertility but most importantly those related to the postnatal well-being of the offspring as reflected in growth [109] or cognitive development [13]. Therefore, follow-up of ART children is highly recommended and is being applied increasingly [11, 60, 82, 94]. Parent-administered questionnaire has been proposed as a way of doing this, since a routine office assessment is costly and time-consuming [78, 92, 121].

The specific concerns in regard to ICSI, whether real or theoretical [20, 23, 24, 30], involve the insemination method, the use of spermatozoa with genetic or structural defects, and the possible introduction of foreign genes. Even if there seems to be no evidence for a higher frequency of congenital abnormalities with ICSI compared to conventional IVF, more subtle effects might become evident in comparison to naturally conceived children. In fact, recent epidemiological studies of ART children report a twofold increase in infant malformations [46], a recurrent reduction in birth weight [108], certain rare syndromes related to imprinting errors [16, 26, 40, 45, 71, 83], and even a higher frequency of some cancers [75]. On the other hand, such observations do not prove that there is an increased risk of imprinting disorders and even less so childhood cancers in ICSI children [30]. A systematic survey aimed at clarifying imprinting issues suggests that only Beckwith-Wiedemann syndrome may have such a link [125].

A shorter gestational age and a lower birth weight have been noted in ART pregnancies irrespective of the conception method [108, 109]. The basis for this could lie in an unclear medical history [55], in the fact that a singleton may result from early in utero loss of a twin [21, 97], or where an ART delivery is performed at the slightest sign of fetal distress [116]. When our Cornell data were stratified according to maternal age, singleton ICSI children had the same mean gestational age and birth weight as those conceived naturally [92]. Similarly, in the study of Lin et al. [63], levels of neonatal distress, NICU admission, and congenital malformations were comparable in both groups. The most unsettling concerns in regard to the health of ART offspring relate to a reported twofold increase in major malformations [46]. However, this study failed to correct for maternal age and for other patient characteristics [122], for the presence of male factor [119], and even in its classification methods [124]. Our follow-up records, beginning in the mid-1990s, have revealed an incidence of malformations within the expected range for the general population of New York State [90]. In another series, ICSI versus naturally conceived singletons that were matched for maternal age displayed no difference in neonatal outcomes [63]; and this was clearly the case when the outcomes of neonates generated by different artificial conception procedure, ICSI versus IVF, were investigated [79].

In this connection, Bowen et al. [13] evaluated the medical and developmental state of 1-year-old children born after ICSI, IVF, or natural conception. They found that most 1-year-old ICSI children were healthy and developing normally, as measured by the Bayley Scales of Infant and Toddler Development. However, about 17% displayed learning difficulties compared to those conceived by IVF or naturally. It was a later report that dismissed this concern in 2-year-old ICSI toddlers [9] which inspired our follow-up study in 3-year-olds.

Because of the confounding role of multiple gestations, as part of a multicenter international investigation, we then decided to assess singleton births at 5 years of age. Although this revealed no differences in the full-scale IQ between ICSI and NC children, surprisingly NC parents displayed higher levels of distress and dysfunctional child interactions and had more difficult children compared to the ICSI group ($P < 0.05$) [99]. This study revealed also that ICSI children were indeed characterized by a lower birth weight ($P < 0.05$) and also by a higher proportion of major malformations ($P < 0.05$) than their NC counterparts. The higher proportion of affected children was concentrated in the Belgian center [10]. Another study comprising 1500 children from 5 European countries failed to show a higher incidence of major malformation in the ART group compared to NC children despite a clear difference in their parental age [11]. The same Brussels group confirmed a malformation level of 10.0% versus 3.4% in the same cohort of NC children once they reached 8 years of age [3]. These three additional malformations included an inguinal hernia and two nevus flammeus that required only minor surgical corrections. Interestingly, when the same ICSI progeny was reassessed for their physical and intellectual performances at 10 years of age, their state matched that of a natural conception group [61]. A prospective match-controlled study was performed to investigate the growth of children in the UK following IVF and ICSI up to 12 years of age. This study showed that height, weight, and head circumference did not differ between IVF and ICSI.

One must also note the emergence of studies evaluating the medical and reproductive health of young adults conceived via ICSI. A recent study compared 54 young men (18–22 years) conceived by ICSI because of male factor infertility to 57 naturally conceived peers and found that the mean levels of FSH, LH, testosterone, and inhibin B were comparable, even after controlling for age, BMI, and season [4]. Although young ICSI-conceived men were more likely to have inhibin B levels <10th percentile and FSH levels >90th percentile compared to naturally conceived peers, these results were not statistically significant [4]. A follow-up study by the same investigators showed that this group of 54 young men had lower median sperm concentration, total sperm count, and total motile sperm count than the naturally conceived peers [5]. However, the small sample size limits the generalization of these findings. The reproductive hormonal profiles of young ICSI-conceived women are currently awaited.

A limited number of studies have addressed the issue of cancer among children conceived by assisted reproduction. A report from the Netherlands identified five cases of retinoblastoma in IVF children – significantly more than expected [75]. A Swedish registry-based study identified five cases of Langerhans histiocytosis among 16,280 IVF infants in comparison to the 0.9 expected cases in the general population [54]. However, these two reports have not been verified by other investigations, and the overall risk of cancer and the individual incidence of all other recorded cancer were not greater than expected. In fact, since childhood cancers are rare, larger studies are required to reliably observe any increase in risk among ART children.

Questions have been posed also about the effect of the embryo culture medium on the health of ART children in general and whether this can induce imprinting disorders [29]. Imprinting disorders such as Angelman's syndrome (AS) [16, 83] and Beckwith-Wiedemann syndrome (BWS) [16, 81, 123] have been described in connection to assisted reproductive technologies. A survey of 1000 British families raising children afflicted with transient neonatal diabetes mellitus, Prader-Willi syndrome, AS, or BWS revealed that only the latter, where *H19*'s downregulation plays a pivotal role, appeared to maintain an association with the ARTs [125]. Both syndromes have been found to be largely due to epigenetic defects rather than a genetic mutation or uniparental disomy [43], thus suggesting that some aspect of the ARTs may be responsible.

In a recent study in mice, Wilson et al. [138] attempted to assess the role of standard IVF and ICSI on gene expression imbalances, and they compared these two procedures to natural mating. The authors concluded that IVF and ICSI were no different in regard to the issues of gene expression but had minor differences observed between the ART procedures and natural conception. Thus, the ART procedure itself does not induce the imprinting imbalances. Another group investigated the basic procedures common to IVF and ICSI such as embryo transfer and in vitro culture [103]. In vitro culture increases the loss of imprinting, but even basic techniques such as embryo transfer can also lead to misexpression of several imprinted genes during post-implantation development.

In summary, the most important factor that can lead to adverse outcomes in offspring conceived by IVF or ICSI is that of high order pregnancies. However, the introduction of single embryo transfer has reduced this considerably. Although perinatal outcomes such as prematurity, low birth weight, perinatal mortality, and increased incidence of malformations have been linked to the techniques of IVF and ICSI, the main culprit is related to infertility itself. Overall, no significant long-term neurodevelopmental differences have been found in connection with the ARTs, though the risks associated with childhood cancer and future fertility still require further research.

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Review Questions

1. What is the least requirement for a selected spermatozoon to provide successful fertilization?
2. Does the presence of a polar body after cumulus removal absolutely indicate ooplasmic maturity?
3. What is the meaning and reason for a three-pronucleated zygote resulting from ICSI?
4. Does assisted fertilization have adverse implications on the health of the offspring?

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Intracytoplasmic Morphologically Selected Sperm Injection

Pierre Vanderzwalmen, Romain Imbert, David Jareno Martinez, Astrid Stecher, Anne Vansteenbrugge, Sabine Vanderzwalmen, Barbara Wirleitner, and Maximillian Murtinger

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Learning Objectives

At the end of this chapter, the readers will be conscious about and able to understand:

- The importance of spermatozoa morphology on fertility.
- Why it is imperative that critical morphological sperm selection criteria are applied before intracytoplasmic sperm injection (ICSI) to improve the chances of embryo development and successful pregnancy with healthy baby outcome.
- That with the introduction of Nomarski optics, subtle sperm head abnormalities such as vacuole-like structures (VLS) are observed.
- That almost 15 years after the first application of motile sperm organelle morphology examination (MSOME) and intracytoplasmic morphologically selected sperm injection (IMSI), selection of spermatozoa devoid of sperm head VLS has multiple benefits.
- That VLS are the morphological manifestation of nuclear dysfunction and represent a pathological situation during the sperm maturation process, which, in turn, may render spermatozoa more vulnerable to DNA damage. This is a strong and tangible argument to select sperm free of nuclear VLS seeing that abnormal chromatin remodeling during sperm maturation could be associated with alteration in the methylation pattern that could affect negatively the prevalence of major fetal malformations.
- Debates and skepticism still exist about the efficiency of IMSI, mainly due to a low number of controlled randomized studies published yet. IMSI is indeed important in women with advanced maternal age, possibly due to reduced correction and reparation capacity of spermatozoon defects in the presence of aging oocyte. In addition, IMSI may prevent absence of blastocyst in case of failure of embryo development to the blastocyst stage in case of previous IVF attempts.
- That the introduction of IMSI made embryologists aware that in times of ICSI, the selection of sperm has to be given proper attention.

In order not to consider IMSI as a frightening technique, practical and technical aspects of sperm selection need to be considered to facilitate the workflow while performing sperm selection before oocyte injection.

33.1 Importance of Spermatozoa Morphology on the Fertility: A Valuable Reason to Improve Its Selection

The introduction of the intracytoplasmic sperm injection (ICSI) 25 years ago permitted to overcome severe male infertility diagnosed by low sperm count and/or poor sperm motility and/or infertility due to morphology deficiency. Sperm morphology – the size and shape of spermatozoon – is one factor that is examined as part of a semen analysis to evaluate male infertility.

The assessment of sperm morphology by Kruger's strict criteria is routinely applied and highlights that sperm morphology analysis of the whole population of a semen sample [1] turns out to be the best predictor of a man's fertilizing potential [2].

A normal sperm has a smooth, oval-shaped head that is 5–6 micrometers long and 2.5–3.5 micrometers wide; a well-defined acrosome cap that covers 40–70% of the sperm head; no visible abnormality of the neck, midpiece, or tail; and no cytoplasmic droplets on the sperm head that are bigger than one half of the sperm head size.

But when we move from the diagnostic level toward patient treatment with intracytoplasmic sperm injection where we have to select only one spermatozoon for injection, the situation is completely different.

The importance of morphologically normal sperm selection is reinforced when facing the reproductive outcomes in terms of fertilization, embryo development, and pregnancy and abortion rates when the oocyte injections can only be done with abnormally shaped spermatozoa (i.e., sperm with elongated, tapered, or amorphous heads, broken necks, or cytoplasmic droplets) [3]. It becomes more and more accepted that abnormally shaped spermatozoa from patients diagnosed with terato- and asthenozoospermia have a significantly increased frequency of aneuploidy, a higher DNA fragmentation index (DFI), and increased rate of mitochondrial dysfunction [4]. With the implementation of restrictive law regulating the number of embryos to transfer, methods of gamete and embryo selection are of paramount importance.

If we keep in mind that ICSI bypasses the natural barriers of reproduction, and knowing the importance of the sperm morphology [3], several novel microscopic approaches, such as differential interference contrast (DIC) microscopy [5], digital holographic microscopy [6], and atomic force microscopy [7], are currently used or in development to assess the morphology of the spermatozoa. They allow a more detailed observation of the different parts of the sperm head (nucleus and acrosome), the midpiece, and the tail.

33.2 Morphological Observation of Living Spermatozoa in Real Time: The MSOME Approach

In order to counteract the problem of morphological evaluation on stained spermatozoa with complex and high-tech systems [6–8], Bartoov [5] introduced, more than 15 years ago, a new innovative noninvasive technique for a more precise morphological evaluation of motile spermatozoa in real time. With the use of Nomarski differential interference contrast (DIC) optics, the so-called motile sperm organelle morphology examination (abbreviated as MSOME) changed the perception of how a spermatozoon suitable for injection should appear. At magnifications ranging from 630x up to 12000x, a more in-depth evaluation of the quality of semen in real time provides substantial information about the normalcy of the head, the neck, and midpiece and in addi-

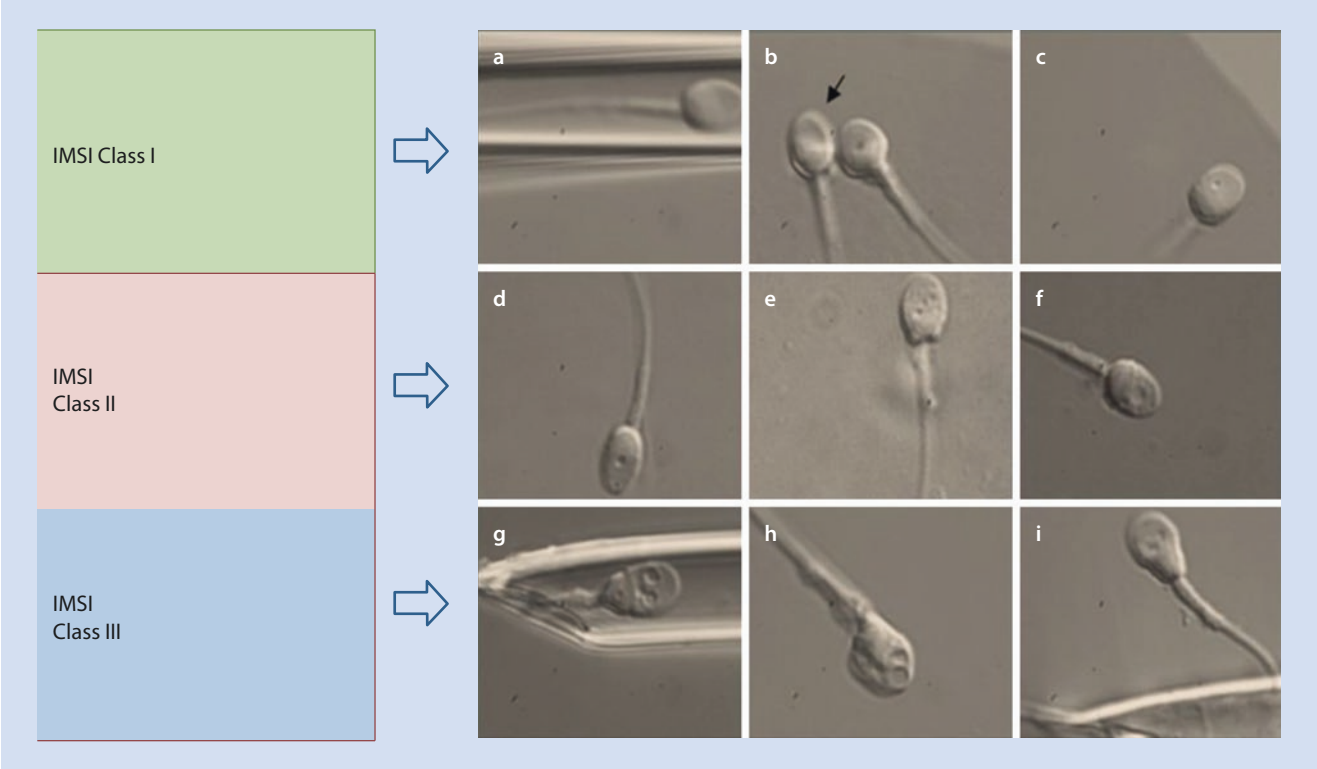

Fig. 33.1 Classification of spermatozoa selected at 6000x

tion allows the detection of subtle sperm head nuclear abnormalities. Morphological assessment at a magnification more than 1000x is obtained after increasing the magnification with a zoom and coupling the microscope with a high-definition digital video camera and a high-definition video monitor.

Bartoov et al. [5] defined the morphological normalcy on motile sperm nucleus according to the shape and chromatin content. The shape has to be smooth, symmetric, and oval with average length and width limits estimated to be 4.75 ± 0.28 μm and 3.28 ± 0.20 μm, respectively. The chromatin mass has to be homogeneous and contains no extrusions or invaginations with a maximum of one vacuole involving less than 4% of the nuclear area.

Regarding the acrosome and the post-acrosomal lamina, they were considered abnormal if absent, partial, or vesiculated. An abaxial neck, with the presence of disorders or cytoplasmic droplets, was considered abnormal, as well as the presence of broken, short, or double and coiled tail.

According to Bartoov [5], normal spermatozoa should not contain nuclear vacuole(s) exceeding more than 4% of the nuclear area. Saidi [9] and Perdrix [10] classified the relative area of the vacuole into three groups: less than 5.9–6.5%, between 6.5% and 13% or between 5.9% and 12.4%, and greater than 13% or 12.4%. For Franco [11], large nuclear vacuoles in spermatozoa were defined by the presence of vacuoles occupying ≥50% of the sperm nuclear area.

In order to estimate the impact of specific sperm defects on embryo development and further outcome in an accurate way, different groups established models of sperm classification according to the normalcy of the shape and the presence and size of vacuoles [12, 13]. Vanderzwalmen [12] classified the spermatozoa into three groups according to the presence and size of vacuoles: Grade I, normal shape and maximum of two small vacuoles; Grade II, normal shape and more than two small vacuoles or at least one large vacuole; and Grade III, abnormal head shapes with or without large vacuoles in conjunction with other abnormalities at the level of the base (Fig. 33.1). Cassuto [13] established a detailed classification scoring in three scales ranging between 6 and 0 points according to the normalcy of the head, the symmetry of the base, and the absence of vacuole.

33.3 Are Sperm Head Vacuoles Authentic Vacuoles?

The morphology of seminal spermatozoa is the end result of a highly complex process of cellular modifications occurring during spermiogenesis. In humans, it leads to widely heterogeneous morphological patterns, with many cellular abnormalities which may be associated with sperm dysfunction [14]. In the WHO classification, one of those defects observed on the head of the spermatozoon is called vacuole. After staining, what is called vacuoles appeared as a clear circle, varying in number, size, and content.

With the introduction of Nomarski optics, subtle sperm head abnormalities such as large or small vacuoles are better

observed. From biological definition, a vacuole present in a cell is a membrane-bound cavity filled with a substance. Boitrelle [7] investigated the structure of vacuoles using atomic force microscopy that permits a three-dimensional scanning of the entire surface of the head of the spermatozoa at high resolution. Boitrelle [7] observed that the sperm plasma membrane was intact but sunken nearby the vacuole and that the sperm head's thickness fell from 700 nm to 300 nm at the site of the large vacuole. They concluded that vacuole-like structures are nuclear depressions which correspond to a concavity in the plasma membrane rather than a hole. Large and small vacuoles were identified as an abnormal, "thumbprint"-like nuclear concavity covered by acrosomal and plasmic membranes.

Using other microscopic optics such as Nomarski differential interference contrast (DIC) optics, other terminologies such as craters [15], concavities [7], hollows [16], or lacunae [17] were assigned confirming that vacuoles appear more as depressions at the cell surface instead of a true vacuole structure that is by definition surrounded by a membrane. As a consequence, the terminology "vacuole" for these structures is misleading and should be better substituted by vacuole-like structure (VLS).

33.4 Pathological Character of Nuclear VLS: A Tangible Argument to Select Sperm Free of Nuclear Defect

33.4.1 Spermiogenesis and Protamine: Histone Transition

During a normal spermiogenesis process, round spermatids undergo four stages, a complex morphological change: successively, the Golgi phase, the cap phase, formation of tail, and the maturation stage. In addition to the visible morphological transformation, the chromatin is radically reorganized and undergoes an extreme condensation resulting in a shift from a nucleosome-based genome organization to the sperm-specific, highly compacted nucleoprotamine structure [18]. During this process, which is essential for the establishment and maintenance of a viable pregnancy, about 85% of human sperm histones are replaced by protamines, whereas only 15% of the DNA remains organized by histones or is attached to the nuclear matrix [19].

These unique cellular reconstruction processes that protect the paternal genome during the transit from the male to the oocyte prior to fertilization require spermatid-specific genes to execute their regulatory roles. It is estimated that 600–1.000 germ cell-specific genes participate in spermiogenesis, and specific genes such as Prm1, Prm2, Tnp1, Tnp2, and H1t2 are involved in chromosomal packaging [20].

Rousseaux et al. [21] and Montellier et al. [22] reported that testis-specific proteins are a new keystone in DNA compaction in humans and murines. One testis-specific protein partially responsible for the replacement of histones by protamines is called bromodomain (BRDT), which possesses two bromodomains capable of interactions with hyperacetylated histones [21]. The genome-wide incorporation of a new histone variant called testis-specific histone 2B (TH2B) might also play an important role in this histone to protamine transition as shown in murine models [22].

Prior to histone replacement by protamines, the nucleosomes are destabilized by hyperacetylation and by DNA methylation level [23]. Crucially, the distribution of the remaining 15% nucleosomes after the 85% nucleosomes to nucleoprotamine replacement is not random but concerns gene regions involved in the epigenetic control and the early embryonic development [19, 23–25]. Repackaging of the haploid genome before delivery in the oocyte is a crucial step. On the other hand, the ratio between the two protamine subtypes protamines 1 and 2, which should normally be close to 1, can have a significant negative impact on fertility when disturbed [26].

Taken together, these data support the idea that bad condensation of sperm DNA has a great impact on male fertility. All these potential epigenetic pattern disturbances may represent the basis of numerous human disorders.

33.4.2 The Hidden Face of VLS

33.4.2.1 VLS and Abnormal Chromatin Condensation

Since the implementation of MSOME to assess sperm morphology, we are more concerned about the presence of large and small VLS and consider them as potential defects [5, 27, 28]. One crucial question to investigate, if we want to implement MSOME in our ICSI procedure, is if VLS are the morphological manifestation of nuclear dysfunction. Assuming that they seem to appear during the last maturation step of round spermatids, do they originate from a natural process or, more likely, from pathological (stress) situations during spermiogenesis or even early in the first stage of the spermatogenesis? In other words, what hides behind spermatozoa mostly with large VLS?

The literature is controversial – while some studies reported that sperm vacuoles should be regarded as a normal feature of the sperm head [16, 29], others mentioned that it is related to male subfertility [30]. However, Tanaka et al. [29] highlighted that the size of the vacuoles is of importance and suggested that spermatozoa with large VLS should not be used for injection. DNA damage may be considered in three different forms: (i) fragmentation of the DNA in the form of single-stranded or double-stranded DNA strand breaks, (ii) nuclear protein defects that may interfere with histone to protamine conversion and subsequent DNA compaction, and (iii) chromatin structural abnormalities causing altered tertiary chromatin configuration [31].

A multitude of studies concluded that vacuoles reveal indirectly nuclear dysfunction in terms of lower mitochondrial potential [32], loss of DNA integrity, higher aneuploidy rate, and problems related to chromatin condensation.

A clear consensus showing a correlation between the presence of VLS and increased aneuploidy and DNA fragmentation

rates was not reported. According to the growing body of literature, a negative correlation between the incidence of vacuoles and abnormally condensed chromatin was observed in all conducted studies [4, 7, 16, 33–38]. In some studies, spermatozoa with or without LVS were selected by micromanipulation before being studied by different microscopy and immunocytochemistry techniques. All the conducted studies concluded first that vacuoles did not take their origin in the acrosome but that they are linked to areas of chromatin decondensation [7]. Moreover, the presence of crater structures most likely reflects molecular defects responsible for anomalies of sperm chromatin packaging and abnormal chromatin remodeling during sperm maturation [27, 28, 39]. Boitrelle et al. and Cassuto et al. [7, 34, 36] observed chromatin condensation at the site of the vacuoles and concluded that large vacuole appears to be a nuclear "thumbprint" linked to failure of chromatin condensation. Perdrix et al. [40] recently published their observations of the correlation between the presence of large nuclear vacuoles and chromosome architecture modifications, adding a new argument for the association between nuclear vacuole-like structure and chromatin disorganization.

We may assume that the integrity of the chromatin is related to the presence or absence of vacuoles in the head of spermatozoa and loss of chromatin compaction renders the DNA more vulnerable to reactive oxygen species (ROS) [41–43].

With the disorganization of the chromatin and the vacuoles in the sperm head, the spermatozoa and its DNA become more assailable to attacks by ROS [34, 45, 46]. Thereby, DNA fragmentation would depend on two steps: the occurrence of vacuoles in connection with insufficient chromatin condensation and the presence of ROS. This could explain why the correlation between the presence of vacuoles and the rate of DNA fragmentation is not observed unanimously seeing that ROS environment is not comparable from one semen sample to another [44, 45].

We may also postulate that when the oxidative stress during spermatogenesis and/or during sperm transport is below a critical level, the DNA and chromatin will not be affected. Above a critical level of ROS, genomic problems start to occur, thereby affecting the reproductive outcome.

33.4.2.2 Potential Consequences of Chromatin Disorganization

Prior to histone replacement by protamines, the nucleosomes are destabilized by hyperacetylation and by DNA methylation level [23]. Crucially, the distribution of the remaining 15% nucleosomes after the 85% nucleosomes to nucleoprotamine replacement is not random but concerns gene regions involved in the epigenetic control and the early embryonic development [19, 23–25]. Repackaging of the haploid genome before delivery in the oocyte is a crucial step.

The spermatozoon delivers a novel epigenetic signature to the egg, required in early embryogenesis that is crucial for normal embryo development [23], and the offspring [44] DNA methylation is a key regulator of transcription and is a major factor controlling imprinted gene expression during embryo development. By analyzing sperm DNA methylation and RNA transcripts in spermatozoa, a recent study showed the paternal contribution and the crucial role of sperm epigenome in embryonic development [46].

Epigenetic patterns are usually faithfully maintained during development. However, this maintenance sometimes fails, resulting in the disturbance of epigenetic processes, which represents the basis of numerous human disorders. Epigenetic modifications in mature spermatozoa play an important role, and, as a consequence, altered methylation profiles may increase the risk of fertilization failure, dysfunction of embryogenesis, preterm birth, low birth weight, congenital anomalies, autism, and perinatal mortality [47–49]. These altered methylation errors are subsequently transmitted to the embryo, conferring a potential risk of imprinting disorders to the offspring [50].

In men with impaired spermatogenesis, the sperm epigenetic landscape is frequently altered. DNA methylation errors and gene expression defects are more frequent in men with abnormal semen criteria than in normozoospermic sperm [51, 52]. Hypermethylation of sperm nuclear DNA could be associated with abnormal semen samples [53] and defective and apoptotic sperm [54]. Epigenetic defects, mainly hypermethylation of certain genes, can lead to altered spermiogenesis and produce poor sperm morphology or defective spermatozoa with a risk of adverse effects on embryo development and increasing risk of imprinting disorders with health implications for the next generation [55, 56].

The fundamental questions to solve are if VLS are related to alterations in the methylation pattern and if potential epigenetic mechanisms could be implied in chromatin condensation failures. In a recent manuscript, Cassuto et al. [57] detected different levels of DNA methylation after morphological sperm selection using the MSOME approach. Sperm DNA methylation level was significantly lower in the normal morphological group compared with the group of abnormal spermatozoa carrying VLS. The same authors previously observed a strong correlation between the presence of nuclear vacuoles and chromatin defects [34]. From these preliminary studies, we may assume that hypermethylation, the source of epigenetic defects, is related to sperm morphology and the presence of VLS.

33.5 MSOME in Combination with ICSI: Intracytoplasmic Morphologically Selected Sperm Injection (IMSI)

The MSOME approach in observing spermatozoa was considered as an additional tool to ICSI and took the name "intracytoplasmic morphologically selected sperm injection" (IMSI) [16, 19, 23–25, 27–58].

Based on previous results of Cassuto et al. [57], deselecting spermatozoon with VLS may effectively predict sperm DNA methylation profiles. IMSI will enable us to discard before injection spermatozoa with a higher risk of methylation aberration due to a predictably poor prognosis and ensure safer biological and clinical outcomes for ICSI.

33.6 Impact of VLS on Early Embryo Development, Pregnancy, and Birth Outcome

The importance of selecting normal spermatozoa becomes obvious when comparing the reproductive outcomes in terms of fertilization, embryo development, pregnancy-abortion rates, and birth outcome when oocyte injections are done with morphologically normal sperms and spermatozoa exhibiting different subcellular defects.

33.6.1 Vacuoles and Embryo Development

In several successive papers [59–63], it was shown that the occurrence of large nuclear vacuoles and/or abnormal shape reduces the percentage of good-quality embryos reaching the blastocyst stage after culture until day 5. Following the outcome of each embryo after injection of spermatozoa, they clearly demonstrated that the use of spermatozoa with no vacuoles or less than two small vacuoles can be associated with significantly higher blastocyst rates than injection with spermatozoa showing more than two small vacuoles or one large vacuole with or without abnormal shape. These studies support the previously issued hypothesis that the impact of male infertility may be at an early stage (early paternal effect) [64], when spermatozoa are not able to attain, penetrate, and/or activate the oocyte, or at a late stage (late paternal effect), when they could not support embryo development, implantation, and pregnancy to term. Late paternal effects are observed after paternal genome activation, and blastocyst development failure is one of their first manifestations [65, 66].

33.6.2 Vacuoles and Pregnancy: Miscarriage

A more specific analysis on the impact of sperm cells with normal nuclear shape but with large vacuoles was first carried out by Berkovitz [27] on two matched IMSI groups of 28 patients each, either with normal nuclear shape but with large vacuoles or with normal nuclear shape spermatozoa lacking vacuoles. No differences in the fertilization and early embryo development up to day 3 were reported. However, injection of spermatozoa with strictly normal nuclear shape but large vacuoles appeared to reduce significantly pregnancy outcomes (18% vs 50%) and seemed to be associated with early abortions (80% vs 7%). Other studies showed also that the selection of normal shape spermatozoa with a vacuole-free head was positively associated with pregnancy and lower abortion rates after day 3 embryo transfers in couples with previous and repeated implantation failures [12, 28, 58, 67–69] and in patients with an elevated degree of DNA-fragmented spermatozoa [28].

Knez [68] showed that there was no significant difference in the pregnancy rates after the IMSI and ICSI procedures and blastocyst transfer. However, after ICSI more pregnancies terminated by spontaneous abortion, whereas after IMSI there were no spontaneous abortions. One explanation could be that IMSI procedure permits to select spermatozoa without defect and, as a consequence, provides more "healthy" blastocysts without chromosomal abnormalities, possibly, in spite of highly comparable development and morphology to ICSI-derived blastocysts.

The link between sperm head vacuoles and impaired chromatin condensation and the occurrence of DNA fragmentation in the presence of ROS may explain why vacuoles can be related to impaired pregnancy outcomes [41, 42, 66, 69, 70].

33.6.3 Vacuoles, Health of Babies Born, and Incidence of Malformations

Up to now, there have not been sufficient numbers of published studies concerning the health of children born after ICSI to draw any firm conclusions about the long-term safety of this procedure. However, it is important to emphasize that animal data are absolutely unequivocal on this point and clearly indicate that DNA damage in the male germ line is potentially hazardous for the embryo and therefore for the resulting offspring [71]. According to a recently published paper, sperm nucleus morphological normalcy, assessed at high magnification, could decrease the prevalence of major fetal malformations in ICSI children [72].

33.7 Vacuole-Like Structure: Acrosomal or Nuclear Origin?

Baccetti et al. reported that nuclear and acrosomal invaginations are formed during spermiogenesis [73]. Based on the classification of Clermont et al. [74], the highest rates of vacuoles (93.8%) are observed when spermatids reach the Sc acrosomal phase [29].

It is obvious that small and large vacuoles are observed in the majority of ejaculated spermatozoa, and their frequency differs according to the severity of male infertility. It is often difficult to observe vacuole-free sperm cells in ejaculates from infertile men, in contrast to semen derived from proven fertile men. According to De Vos et al. [75], the prevalence of vacuoles in normally shaped spermatozoa seems to be low. According to Silva et al. [76] and De Almeida Ferreira Braga et al. [77], the frequency of large nuclear vacuoles was significantly higher in advanced paternal age compared to the younger age groups.

For Kacem et al. [78] and Montjean et al. [79], vacuoles are mostly of acrosomal origin. Vacuoles are mainly filled with acrosomal enzymes such as trypsin-like acrosin that may induce a harmful effect after oocyte injection [78]. As a consequence, they concluded that a large majority of normal, regularly shaped spermatozoa showing no vacuoles have already undergone their acrosome reaction and should be selected for injection. This could be true if vacuoles were a completely closed entity. But seeing that VLS

are considered as a depression and not as a closed structure, it is improbable that such hypothesis and mechanism reflect the reality.

Montjean et al. [79] tested the effect of inducers of the acrosome reaction. After incubation of sperm in either hyaluronic acid or follicular fluid for 90 min, they observed a highly significant decrease in the presence of vacuoles as a consequence of the acrosome reaction.

On the semen of two men suffering from globozoospermia, Gatimel et al. [80] confirmed, by transmission electron microscopy (TEM) and scanning electron microscopy (SEM), that vacuoles were present in the majority of spermatozoa at a rate comparable to that observed in fertile controls, even though the totality of spermatozoa lacked acrosomal structures. Neyer et al. [81] monitored the same population of spermatozoa in a sperm capture channel for 24 h and observed that the induction of the acrosome reaction using calcium ionophore A23587 did not lead to any modifications in preexisting vacuole appearance, disappearance, or formation.

We may conclude that there is a negative relation between the presence of vacuoles and the sperm capacity to undergo acrosome reaction. According to Boitrelle et al. [7, 36], the sperm membrane and the acrosome cap are intact at the site of these depressions. Likewise, Perdrix et al. [33] demonstrated an exclusive nuclear origin of these large head surface depressions using TEM supporting their severe impact on sperm quality.

Such studies reinforce the opportunity to identify and discard prior to injection low-quality spermatozoa carrying nuclear vacuoles, which have a higher risk of DNA hypermethylation [57].

33.8 Indications for IMSI

As we mentioned previously, there is a real benefit to selecting morphological spermatozoa free of vacuoles. But, for different reasons, there is undeniable evident skepticism about this method of spermatozoa selection. Different doubts and interrogations may justify the hesitations to apply IMSI.

After several years, the superiority of IMSI over ICSI is still a matter of debate, and even to date randomized and well-powered studies to confirm a benefit of IMSI are limited – and even depict conflicting results [82].

According to the literature, controversial conclusions have been drawn, especially in terms of fertilization, top-quality embryo rates, and pregnancy, after comparing IMSI and ICSI. Some studies have shown that IMSI improves reproductive outcomes in cases of male factor infertility and/or previous failed ICSI attempts in terms of implantation and clinical pregnancy rates as compared with conventional ICSI [28, 58, 59, 68, 83–87, 88, 89]. On the other hand, IMSI and conventional ICSI seemed to provide comparable laboratory and clinical results when an unselected infertile population was evaluated [75, 89] or when IMSI was applied as the first treatment option [90].

33.8.1 Full Benefit of IMSI Is Attained with Blastocyst Culture

One of the main benefits of IMSI is the higher rate of blastocysts obtained per cycle when morphologically good-quality spermatozoa are selected [13, 59, 61, 68]. Nowadays, with successful vitrification techniques, the full benefit of a better spermatozoa selection is highlighted, if we are able to produce one more blastocyst per cycle. In fact, more blastocysts provide higher chances for the patient to achieve pregnancy in successive vitrified-warmed embryo transfer cycle(s).

33.8.2 MSOME Selection in Globozoospermia to Circumvent Artificial Activation

Globozoospermia is a male factor infertility that affects men because several abnormalities of sperm remodeling during spermiogenesis will entail to formation defects or premature elimination of acrosomal structures. Fertilization capacity after ICSI is low mainly due to a lack of sperm-specific phospholipase C (PLCzeta) that is responsible for inducing Ca2+ oscillations essential for oocyte activation [91, 92].

At present, births have been reported after ICSI followed by artificial Ca2+ oscillation induction via the use of assisted oocyte activation protocols by exposing oocytes to ionophore [93–95].

Another approach proposed by Gatimel [80] and Kashir [96] is to apply MSOME to maximize the efficacy of ICSI/IMSI. Sermondade [97] selected spermatozoa that exhibited a small acrosome which was not visible using conventional sperm selection methodology. Gatimel identified those with some sparse oval forms revealing the presence of Golgi residues [80]. These authors showed that some globozoospermic spermatozoa possess an acrosomal bud that contains a total amount of PLCzeta that is not significantly different from fertile controls and which correlates to successful oocyte activation and fertilization without the need for artificial oocyte activation with exogenous chemical agents.

33.8.3 IMSI and Advanced Maternal Age

Injection of a morphologically normal spermatozoon overcomes the low oocyte quality in older women, resulting according to Setti [98] in improved embryo quality and in a ninefold increase in the clinical pregnancy rate. Our results (unpublished data) corroborate the data of Setti [98] that semen quality seems to be more important with advancing maternal age, a group of patients that are of big relevance in assisted reproductive technology (ART). The rate of single blastocyst transfer (SBT) with top-quality blastocysts in the younger women group (<38 years) was 56.9% when morphologically normal spermatozoa were selected and 54.2% with spermatozoa carrying large VLS. On the other hand, for

women aged ≥38 years, the rate of SBT was 31.0% with normal spermatozoa and 25.0% ($p < 0.01$) with spermatozoa carrying large VLS.

Regarding the birth rates (BR) per SBT, IVF success was impaired in the higher age groups after injection of sperm with VLS. In the younger group, BR of 45.1% and 54.2% were obtained, respectively, after injection of normal and VLS spermatozoa. In contrast, a significant decrease in the BR is observed, 31.0% (normal Sp.) versus 12.5% (Sp. with VLS), in the advanced maternal age group.

Blastocysts derived from fertilization of sperm with VLS and/or suboptimal morphology decreases chances for a live birth in women >38 years probably because of reduced correction and reparation capacity of the aging oocyte.

33.8.3.1 DNA Damage-Repairing Factors

If we assume that a majority of nuclear defects reflected in vacuoles may be correlated with DNA integrity, we may have an optimistic view in the sense that this damage brought into the zygote by the fertilizing spermatozoon may be effectively repaired by oocyte factors [99–101]. We may then postulate the possibility to obtain blastocysts that will further develop into a healthy baby even though the oocyte was injected with a spermatozoon carrying a large vacuole. This aspect is related to the tolerance of the oocytes toward DNA decays [99], especially if young and/or good-quality oocytes are capable of repairing and rescuing the DNA of poor-quality spermatozoa. As a consequence, even if the fertilizing spermatozoon carries DNA damage in its genome, the oocyte could repair this damage, and therefore, it would be of no consequence for embryo and fetal development.

However, we cannot determine whether the oocyte would be capable of repairing this damage. In fact, oocytes can repair sperm DNA damage; however, a threshold exists beyond which sperm DNA cannot be repaired. This mainly depends on the degree and the type of sperm DNA damage (single-stranded vs double-stranded damage) [102]. Also, oocytes whose DNA repair mechanisms are not functional (aging oocytes – in vitro culture conditions) or have been damaged by endogenous (e.g., free radicals) or exogenous (e.g., radiation and environmental toxicants) factors might not be able to repair this damage.

33.8.4 Limitation of IMSI: Semen with Only Morphologically Abnormal Spermatozoa with VLS

There is a great heterogeneity between all the semen samples. Thus, the frequency by which good spermatozoa can be selected varies greatly from one patient to the other. In some cases, only one part of the oocytes can be injected with morphologically normal spermatozoa. For other patients, as already noticed earlier by Berkovitz et al. [27, 83], in spite of having a more powerful selection method at hand, it is not always possible to find and select morphologically completely normal-appearing spermatozoa for injection, even after extensive search. In such situations, is IMSI helpful? We have observed, out of 1146 MSOME analyses, a low frequency (#14, 1.2%) of semen samples with 100% abnormal shape and VLS. After 14 IMSI cycles, four pregnancies were noticed, resulting in one birth (7.1%) in a woman of 33 years old.

33.9 IMSI in 2017: Still a Debate

Several reservations concerning the technical aspect, organization, and injury of the gametes were arguments against applying this method of spermatozoa selection.

Three critics about IMSI are frequently argued. First, some argue that exposing the spermatozoa for a longer period at 37°C increases the rate of VLS. The second one concerns the prolonged period of time for oocytes out of the incubator during spermatozoa selection. The third criticism is the point that the prolonged selection time of male gametes may be at the detriment of oocyte and promotes oocyte aging. In addition, other criticisms such as it is a time-consuming technique and requires a very sophisticated, complicated, and high-cost system are frequently evoked.

33.9.1 In Vitro Formation of Vacuoles: A Reality at 37°C

Peer et al. [103] concluded that after 2 h of incubation at 37°C in culture media, the incidence of spermatozoa with vacuolated nuclei was significantly higher, so that prolonged sperm manipulation for assisted reproduction therapy should be performed at 21°C rather than 37°C.

Neyer et al. [81] using the sperm-microcapture channels technique observed that neither incubation at 37°C nor induction of oxidative stress induces de novo formation of nuclear vacuoles. According to these observations, they concluded that nuclear vacuoles on the sperm head are already produced at earlier stages of sperm maturation and are not induced or modulated by routine laboratory procedures [104]. Schwarz et al. [105] concluded that sperm nuclear vacuolization is unaffected by temperature in motile sperm isolated by swim-up.

33.9.2 How to Facilitate the Workflow While Performing IMSI

33.9.2.1 First Approach

In order to reduce either the time for the oocytes out of the incubator or the selection time of spermatozoa to avoid oocyte aging, a practical and technical approach is implemented. Spermatozoa are first selected using the Nomarski DIC optics and then injected on the conventional Hoffman ICSI microscope [106].

With such a strategy:

1. The oocytes are not present in the IMSI dish during selection of the spermatozoa. After selection, the dish is incubated for 15–30 min in order to stabilize the temperature (T°) and/or the pH value (in case no HEPES buffer culture medium is used).
2. In case of severe teratozoospermia, taking into account that more time for sperm selection is needed, we plan the selection at least around the time of oocyte pick-up (OPU). With such a policy, oocyte injection can be performed 2–3 h after OPU (38–40 h post hCG administration) on the ICSI station, avoiding oocyte aging.
3. The IMSI microscope is not occupied for an excessive period, and finally the oocytes are removed from the incubator only for the injection step. In case of several IMSI, the IMSI station is only occupied for the selection process and not for the injection phase, causing a better organization of the laboratory work.

Under sterile conditions, several drops are deposited in a glass bottom dish (Fig. 33.2). Two dishes are prepared when more than two mature oocytes are available.

A small aliquot of washed sperm (1–10 µl according to the semen sample quality) is deposited into drop A (Fig. 33.2a) The motile spermatozoa swim into the long "snake shape" drop. The morphologically normal ones and the best second class are selected, at 1000x magnification, with an ICSI pipette. The selection starts first at the end of the drop (A1). The selected motile spermatozoa are transferred in the host-selected spermatozoa microdrop (C). Release carefully the spermatozoa in order to keep them motile until the time of ICSI. In case of severe asthenozoospermia, the sperm aliquot is deposited at least 1 h before starting selection in order to give time to the spermatozoa to swim out in the "snake drop."

After collecting spermatozoa (if possible, 1.5 times the number of oocytes to inject), the dish is removed from the IMSI station and placed on a 37°C heating stage for temperature recovery (~30 min).

After this period of incubation at 37°C, the oocytes are placed into the culture media (D), and ICSI is performed using a conventional Hoffman microscope at 400x magnification (Fig. 33.2b). Motile spermatozoa are aspirated from the preselected host drop (C) and immobilized in the PVP drop (B) before injection.

33.9.2.2 Second Approach

In a second approach, the selection and injection are performed exclusively on the Nomarski DIC microscope. As previously described (Fig. 33.2), a minimum of two dishes are prepared except that sperm host drops (C) are not prepared.

Selection and injection are realized:

1. When few IMSI cycles are planned, so that the IMSI microscope will not be occupied for a too long period.
2. When the purpose of the study is to follow the outcome of embryo development in relation to the type of injected spermatozoon (photo documentation of the injected spermatozoon).

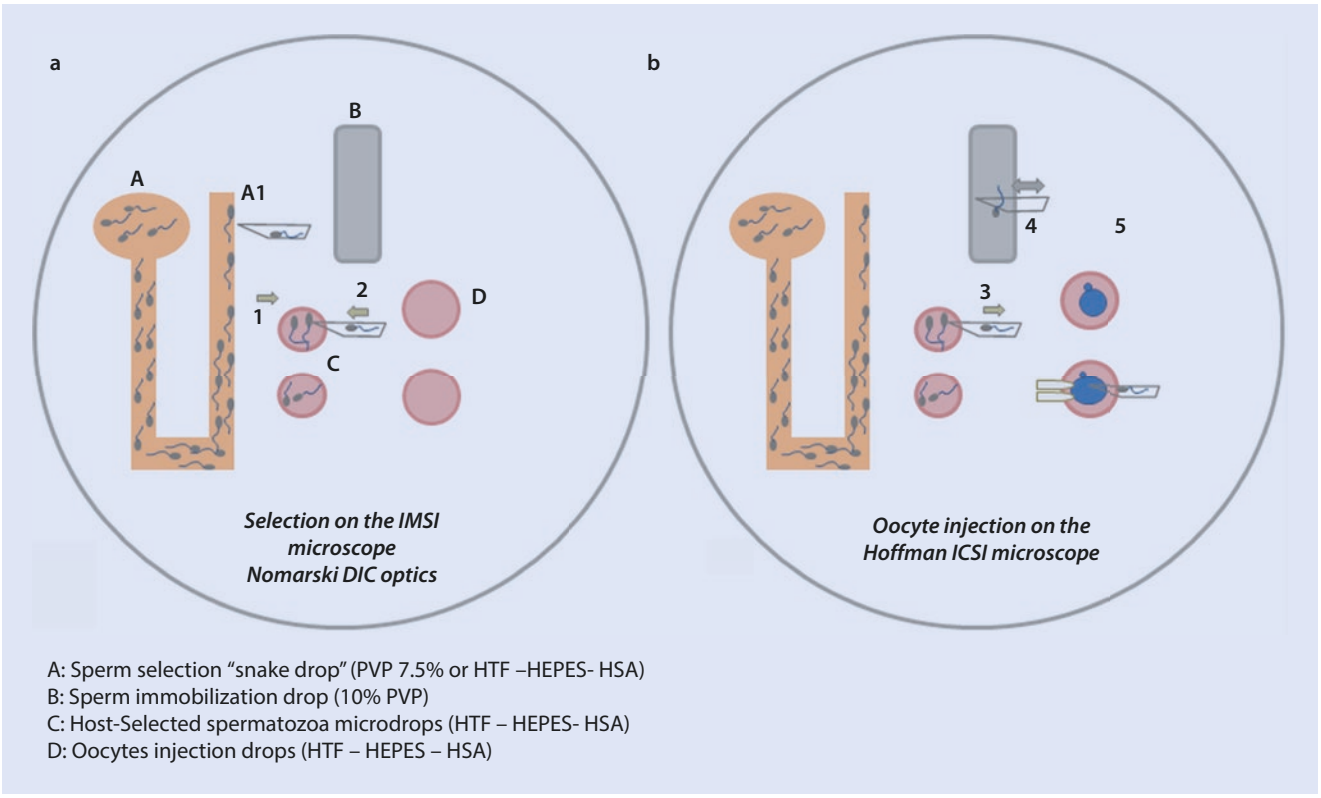

Fig. 33.2 Sperm selection with Nomarski DIC optics **a** and oocyte injection on the Hoffman ICSI microscope **b**

With such a strategy a maximum of two oocytes are deposited in drop D. But before applying this approach, the time spent to select one spermatozoa of class I or II has to be assessed. The primary intention is to choose normally shaped spermatozoa without any vacuoles (class I) for injection into the oocytes. Depending on the degree of impaired sperm morphology, the mean time required for selecting the best sperm ranged between 2 and more than 10 min.

When the time does not exceed 2–3 min per spermatozoon, then two oocytes are deposited in the drop. For a period ranging between 3 and 10 min, oocytes will be injected one at a time. In case the time exceeds 10 min, then the first approach is realized.

Moreover, when it is obvious after 10 min of sperm examination that it is not possible to select class I spermatozoa, the second-best spermatozoa with the least number of vacuoles and/or other abnormalities are selected for injection.

33.10 Conclusion

One of the most essential questions is not under which technical conditions the selection of spermatozoa should be recommended but rather if we have to consider selecting the best spermatozoa and, if possible, excluding those carrying defects such as vacuoles.

It is increasingly obvious that large vacuoles, which are concavities in the sperm head, reflect a pathological situation. VLS first appear during the spermiogenesis, rendering the nucleosome and DNA and connected molecules more vulnerable to intrinsic or extrinsic attacks by ROS. According to the level of ROS, DNA fragmentation may appear.

The literature reveals a clear significant correlation between sperm head morphology and DNA methylation profile. High-magnification visualization of sperms in real time provides the opportunity to identify and discard low-quality spermatozoa, which have a higher risk of DNA hypermethylation, prior to injection, ultimately improving ART outcomes by decreasing the risk of birth defects, major malformations, and epigenetic diseases either in the offspring or for the future generation through ICSI.

Considering the thesis of reduced reproductive outcome due to DNA damage in sperm and the notion that such DNA damages are displayed by the formation of VLS which are accurately evaluated at high magnification by MSOME, we may suggest that the more sophisticated selection of spermatozoa using the MSOME approach will most probably substitute the classical ICSI sperm selection method within the next years.

IMSI is for sure an indication for older patients. Semen quality seems to be more important with advancing maternal age. Blastocysts derived from fertilization of sperm with VLS and/or suboptimal morphology decreases chances for a live birth in women >38 years. This is most possibly due to reduced correction and reparation capacity of the aging oocyte. Thereby the application of IMSI leads to more blastocysts of higher quality, increasing the chances to transfer an embryo with a high implantation potential and to achieve the birth of a healthy baby.

Seeing that this simple, noninvasive technique still raises debates and skepticism about its efficiency, mainly due to a low number of controlled randomized studies published yet, one fundamental question is whether we should – with the knowledge that sperm VLS are related to abnormal chromatin packaging and possibly to DNA fragmentation – select spermatozoa with these defects for injection if we have only to change the optics. As far as we know, there is no reason for not selecting the morphologically best spermatozoa. Why therefore perform a spermocytogram as the first exam if there is no belief in morphological sperm selection?

Review Questions

1. You want to implement a low cost IMSI station:
   Which equipment is mandatory to observe precisely and easily spermatozoa with small or large vacuoles?
   High magnification system
   or
   Nomarski differential interference contrast (DIC) optics

2. Why should spermatozoa with large VLS be deselected for injection?

3. Why is repackaging of the haploid genome before delivery in the oocyte a crucial step?

4. In which situations is it better to apply IMSI?
   Previous attempt with no fertilization
   Previous attempt with fragmented embryo development
   Woman aged older than 38 years

5. You have to face the following situations: woman with 15 follicles and man with one million spermatozoa. How will you organize chronologically your work?
   Oocyte retrieval – sperm preparation – dish preparation – sperm selection and isolation of the best-looking spermatozoa – IMSI
   or
   Sperm preparation – dish preparation – sperm selection and isolation of the best-looking spermatozoa – oocyte retrieval – IMSI

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Oocyte Activation Deficiency and Advances to Overcome

Marc Yeste, Celine Jones, Siti Nornadhirah Amdani, and Kevin Coward

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M.Y. wrote the chapter. C.J., S.N.A. and K.C. critically revised the chapter and approved the final version.

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Learning Objectives

- To learn what oocyte activation is and when it does occur
- To understand the mechanisms underlying oocyte activation and to emphasize the crucial role of a sperm-specific protein, PLCζ
- To comprehend the relationship between oocyte activation deficiency and human infertility
- To obtain a global picture of current ART that allows artificial activation of the oocyte

34.1 What Is Oocyte Activation?

Upon ovulation, oocytes have not yet completed the second meiotic division and remain arrested at metaphase II (M-II; see Tripathi et al. [1] for review) until the oocyte is fertilized by a spermatozoon [2, 3]. The process by which the oocyte resumes the second meiotic division is known as "oocyte activation" [4, 5]. During this process, a sperm cell triggers a series of calcium (Ca^{2+}) oscillations within the ooplasm which are involved in crucial events, such as the exocytosis of cortical granules, extrusion of the second polar body, regulation of gene expression, and the initiation of embryogenesis [4, 6, 7].

Although substantial progress has been made over the last decade, the precise mechanism by which the presence of a sperm within the ooplasm triggers the process of oocyte activation remains debatable, and the identity of the sperm factor responsible for this process has been the source of much scrutiny [8]. During the 1980s, three theories were proposed to explain this event (reviewed by Machaty [5]): (1) the "receptor" theory, which proposed that a sperm ligand interacted with a receptor situated on the oocyte plasma membrane and that the oocyte was activated upon this interaction [9]; (2) the "sperm factor" theory, which hypothesized that oocyte activation was triggered by a catalytic substance residing within the sperm head which entered the ooplasm following gamete fusion [10]; and (3) the "calcium bomb" theory, which proposed that sperm acted as a conduit to transport Ca^{2+} into the oocyte [11]. Of these, the "sperm factor" and "receptor" theories were the most credible. However, the "receptor" theory was readily dismissed when the first intracytoplasmic injection (ICSI) attempts demonstrated that oocyte activation was possible even when a potential ligand-receptor binding mechanism involving the gamete membranes was completely bypassed [12]. In support of the "sperm factor" theory, studies published in the 1990s demonstrated that Gq proteins were not associated with sperm-mediated oocyte activation [13] and that the microinjection of soluble sperm extracts into the oocyte cytoplasm successfully triggered Ca^{2+} oscillations [14, 15]. Progress during this vital era was fueled by developments in our ability to image changes in the Ca^{2+} content of oocytes in response to experimental test substances, a technique which was instrumental in demonstrating the ultimate suitability of the "sperm factor" theory [16], which remained as the only credible theory.

34.2 The Sperm-Borne Oocyte Activation Factor

34.2.1 The First Attempts

The identity of the sperm-borne oocyte activation factor (SOAF) has been the target of several researchers, and, from a historical point of view, different candidates have been suggested.

In 1996, Parrington et al. [14] found that glucosamine-6-phosphate deaminase (GNPDA), a protein referred to as "oscillin," triggered Ca^{2+} oscillations in hamster oocytes following fertilization. However, subsequent attempts to confirm these findings failed as neither the microinjection of recombinant oscillin nor its human and porcine homologues were able to trigger Ca^{2+} oscillations [17–19]. As a result, oscillin was quickly dismissed as the SOAF.

Following oscillin, five other putative sperm factors have been reported for vertebrates in the literature. It should, however, be emphasized that some of these candidate proteins were proposed from studies performed in nonmammalian species.

34.2.2 The Truncated Form of CD117 (TR-KIT)

One suggested candidate was the truncated form of CD117, the mast/stem cell growth factor receptor (SCFR) also known as c-kit tyrosine kinase receptor (TR-KIT). This protein resides in equatorial and postacrosomal sperm regions and was reported to release M-II arrest in mouse oocytes, induce cortical granule exocytosis, and be involved in the formation of pronuclei [20–22]. Although the mechanism of action for this protein is not yet fully understood, it has been suggested that CD117 interacts with FYN, a Src-like tyrosine kinase present in the oocyte cortex [158]. Following this interaction, it was postulated that CD117 would be cross-phosphorylated by FYN and that the CD117/FYN complex would interact with PLCγ1, an oocyte phospholipase [22, 23], which, in turn, would initiate the typical Ca^{2+} oscillation pattern. While clinical evidence points to other SOAF candidates, and the mechanism of action of CD117 is not fully understood, some studies have indicated that the presence of CD117 in human sperm is correlated with DNA integrity and that sperm from globozoospermic patients exhibit lower amounts of this protein relative to normal sperm from fertile men [24, 25].

34.2.3 Cytrate Synthase

Work conducted in amphibians by Harada et al. [26] led to the suggestion that cytrate synthase (CS) could represent a SOAF candidate. This 45 kDa protein is found in the connecting and intermediate pieces of the sperm from *Cynops pyrrhogaster* and is able to activate the eggs of this species. However, it was not possible to ascertain the precise role that

this protein plays, as other suggested SOAF candidates, such as mouse phospholipase C zeta (PLCζ), are also able to activate amphibian eggs. Moreover, no clinical data are available for this protein in human spermatozoa, and researchers thus directed their efforts toward other SOAF candidates.

34.2.4 Postacrosomal Sheath WW Domain-Binding Protein

One of the SOAF candidates that has attracted much attention recently is the postacrosomal sheath WW domain-binding protein (PAWP, also known as WBP2NL). This protein resides in the sperm perinuclear theca at the postacrosomal sheath level [27, 28], a region that has been suggested by different authors to be crucial for the activation of the oocyte [29–31].

With regard to how PAWP works, this protein presents a consensus PPXY sequence which binds WW group I protein domains, from which the name is derived. For this reason, the PPXY domain of PAWP has been postulated to interact with the WW group I protein domain from an oocyte PLCγ, which would be directly involved in the onset of Ca^{2+} oscillations [32]. Related to this, another PLC oocyte isoform (PLCβ1) has been reported to be involved in Ca^{2+} oscillations, as a reduction in the levels of PLCβ1 in mouse oocytes results in Ca^{2+} oscillations of lower amplitude.

The literature remains inconsistent with regard to the exact role of PAWP during oocyte activation (reviewed by Amdani et al. [8]). Indeed, different studies have indicated that the microinjection of recombinant PAWP, or protein extracts from the perinuclear theca (where PAWP resides), into the oocytes of *Xenopus*, pig, cattle, macaque, and humans allowed M-II oocytes to conclude meiosis II [27, 32, 33]. In addition, PAWP levels evaluated through immunofluorescence in bull and human sperm were related to their fertilizing ability when assessed following in vivo fertility trials and ICSI, respectively [34–36]. Yet, sperm from globozoospermic men present significantly lower levels of PAWP than those from fertile men [25].

In contrast, Nomikos et al. [37, 38] reported that PAWP failed to trigger Ca^{2+} oscillations when injected into mouse oocytes, and Satouh et al. [39] found that a knockout mouse model for PAWP was able to trigger normal Ca^{2+} oscillations and give rise to normal embryonic development. Therefore, at present, PAWP does not appear to represent a credible SOAF candidate as it does not trigger Ca^{2+} oscillations, but clinical data indicate that it could be involved, at least to some extent, in other events occurring at the beginning of embryogenesis.

34.2.5 Phospholipase Cζ (PLCζ)

Mounting evidence currently indicates that PLCζ (1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase zeta-1) is the most credible SOAF candidate responsible for oocyte activation [40–44], as microinjection of either recombinant PLCζ or complementary RNA (cRNA) encoding for PLCζ triggers Ca^{2+} oscillations, causes cytoplasmic movements coincident with Ca^{2+} oscillations, and allows embryogenesis to begin [45–48]. As with any PLC, PLCζ hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG; [49]). Inositol 1,4,5-trisphosphate then interacts with its receptor (IP_3R), residing on the endoplasmic reticulum (ER) membrane within the oocyte. This induces the release of Ca^{2+} from the ER into the cytosol and Ca^{2+} oscillations begin to occur. Diacylglycerol activates protein kinase C, which also depends (at least in the case of conventional isoforms) on the Ca^{2+} released from the ER [50, 51].

It is worth mentioning that PLCζ is a sperm-specific PLC isoform, which not only is the smallest PLC (70.4 kDa in human sperm) but does not function in the way that other PLCs do. Indeed, PLCζ is not located on the plasma membrane of oocytes and sperm and is not linked to G-protein-coupled receptors (GPCRs). In addition, PLCζ does not hydrolyze PIP_2 residing in the plasma membrane, but rather appears to target stores of PIP_2 residing in specific oocyte vesicles distributed across the ooplasm [52, 53].

Although the exact mechanism of action for this molecule remains unknown, several attempts have allowed us to shed light on this particular issue through the use of PLCζ chimeric forms. In terms of structure, PLCζ presents four EF hand domains (a helix-loop-helix domain typical of Ca^{2+}-binding proteins) and, a C2 domain, along with catalytic X and Y core domains (Fig. 34.1). In contrast to other PLCs, PLCζ does not possess a PH domain, which is the domain that normally targets the PIP_2 residing in the plasma membrane [55, 56]. EF1 and EF2 domains are involved in protein activity [57], while EF3 is a Ca^{2+}-sensitive domain [58]. The C2 domain interacts with phosphatidylinositol 3-phosphate and phosphatidylinositol 5-phosphate but not with PIP_2, which reduces PLCζ activ-

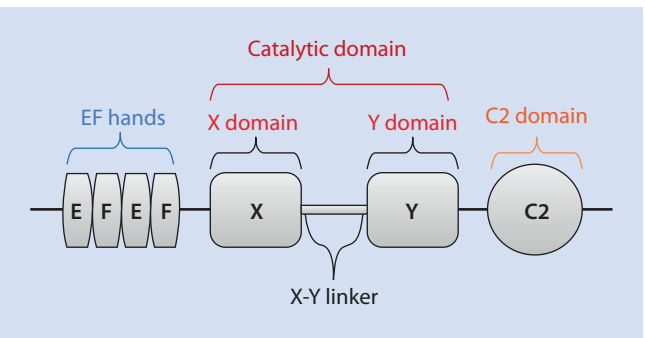

Fig. 34.1 Schematic representation of the PLCζ structure. This protein presents four EF hand domains (a helix-loop-helix domain typical of Ca^{2+}-binding proteins) and a C2 domain, along with catalytic X and Y core domains. The X-Y linker region targets PIP_2. (Reproduced from Ramadan et al. [54] under the terms of the Creative Commons Attribution License 4.0)

ity [57]. The X-Y linker region targets PIP_2 and confers maximal enzymatic activity [53, 59, 60].

Phospholipase Cζ has been identified in the sperm of humans and other mammals, such as monkeys, hamsters, pigs, and horses [40, 43, 61–64], and in nonmammalian species [65, 66]. With regard to its localization, PLCζ is mainly found in the equatorial and postacrosomal regions of ejaculated spermatozoa [67–69]. Immunogold and transmission electron microscopy have also provided definitive evidence that this protein resides in the perinuclear theca [41]. Following capacitation and the acrosome reaction, PLCζ is mainly localized to the postacrosomal sperm region [70].

As mentioned earlier, PLCζ is, at present, the strongest SOAF candidate. Most of this credibility comes from the analysis of clinical data, which points to a relationship between PLCζ and male infertility. In effect, proportions of sperm exhibiting PLCζ are significantly higher in fertile men than in infertile patients and are correlated with fertilization rates following ICSI [68, 71]. In addition, abnormal localization and mutations in PLCζ have been associated with oocyte activation deficiency (OAD) and total fertilization failure [48, 72–79]. Furthermore, the absence of PLCζ or deficient forms of PLCζ do not appear isolated in some cases but are concomitant with other anomalies. This is the case of globozoospermia, a rare and very adverse condition in which sperm are morphologically abnormal, exhibit high DNA fragmentation levels, and possess little or no PLCζ [25, 80–83].

In spite of all these observations, some researchers have challenged and raised concerns over the suitability of PLCζ as the principal SOAF. For example, Aarabi et al. [84] concluded that PLCζ was not present in the perinuclear theca, the sperm compartment in which the SOAF is supposed to reside [29–31], and observed PLCζ staining following the acrosome reaction and sperm-oocyte membrane fusion. Furthermore, these authors reported that rather than being present in spermatids at the end of spermiogenesis, PLCζ was secreted by principal epididymal cells [84]. In contrast to the work by Aarabi et al. [84], Escoffier et al. [41] did identify PLCζ in the perinuclear theca of human sperm, and Castillo et al. [85] observed PLCζ in the soluble chromatin fraction of the sperm head, which supports the idea that this protein arises during spermatogenesis. Given that the specificity of antibodies is always a matter of concern, as different antibodies may provide different results, more research is warranted to confirm the crucial role of PLCζ, despite the supportive evidence obtained thus far. Finally, a knockout mouse model for PLCζ has recently been produced through the CRISPR/Cas9 gene editing tool [86]. Interestingly, while sperm from these *Pcz1*$^{-/-}$ males were unable to trigger Ca^{2+} oscillations in mouse oocytes, which supports PLCζ as the most credible SOAF candidate, those males were subfertile rather than sterile, and oocyte fertilization, although in a less efficient manner (higher polyspermy), occurred in the absence of PLCζ [86]. For this reason, it is prudent to suggest that other proteins and/or alternative routes may be involved in oocyte activation.

34.3 The Sequence of Events During Oocyte Activation

After intercourse, sperm are deposited in the vagina and traverse the cervical mucus, which works as a selection barrier. Spermatozoa then move toward the fallopian tube [87]. It is not yet clear whether a sperm reservoir exists in the isthmus in humans, as proven for other mammals with an estrous cycle [88, 89]. In any case, what is clear is that sperm must become capacitated before being able to fertilize the oocyte and that this process also takes place within the fallopian tubes [90, 91]. Following capacitation, spermatozoa are able to trigger acrosome exocytosis [92].

While it has been widely reported that sperm trigger the acrosome reaction upon interaction with the proteins within the zona pellucida (ZP), recent data indicate that this event could take place earlier, in response to progesterone secreted by the cumulus cells [93–95]. The acrosome reaction allows the sperm to pass through the ZP. Following this, binding between the sperm and oocyte plasma membranes occurs, involving several proteins such as sperm proteins from the ADAM family, oocyte protein CD9, and other proteins which have yet to be identified [96–98]. It has been demonstrated that IZUMO1, a protein located in the equatorial sperm region, interacts with JUNO, its oocyte receptor, and that this binding is critically involved in membrane fusion [99–101].

After membrane fusion, the sperm is engulfed by the oocyte cytoplasm and PLCζ protein diffuses, via a yet unidentified mechanism which involves a PLC/phosphoinositide transduction pathway, across the ooplasm for approximately 10 min [102, 103]. PLCζ then targets PIP_2 molecules residing in oocyte vesicles distributed across the ooplasm rather than those present in the inner oolemma leaflet and initiates the first calcium wave within 30 min of gamete fusion [53, 104]. Given the specificity of PLCζ for PIP_2 residing in oocyte vesicles, Swann and Lai [104] raised the hypothesis that PLCζ is likely to interact with one or more than one oocyte-specific protein located on the membrane of these vesicles. This would explain why PLCζ hydrolyzes PIP_2 molecules of these vesicles and not those present in the oocyte plasma membrane (Fig. 34.2).

PLCζ hydrolyzes PIP_2 into IP_3 and DAG. Inositol trisphosphate is then released from the oocyte vesicle, diffuses across the ooplasm, and interacts with its receptor (IP_3R), a ligand-gated channel present in intracellular Ca^{2+} stores such as the endoplasmic reticulum, via two phosphate groups which bind to opposite sides of the IP_3-binding site (Fig. 34.2). This interaction triggers a conformational change which opens the channel, allows the Ca^{2+} to be released from the stores, and initiates the typical pattern of Ca^{2+} oscillations [106]. As a ubiquitous secondary messenger, Ca^{2+} intervenes in many events, regulates the activity of several proteins which are involved in the release of M-II arrest, and coordinates movements in the oocyte cytoplasm which take place

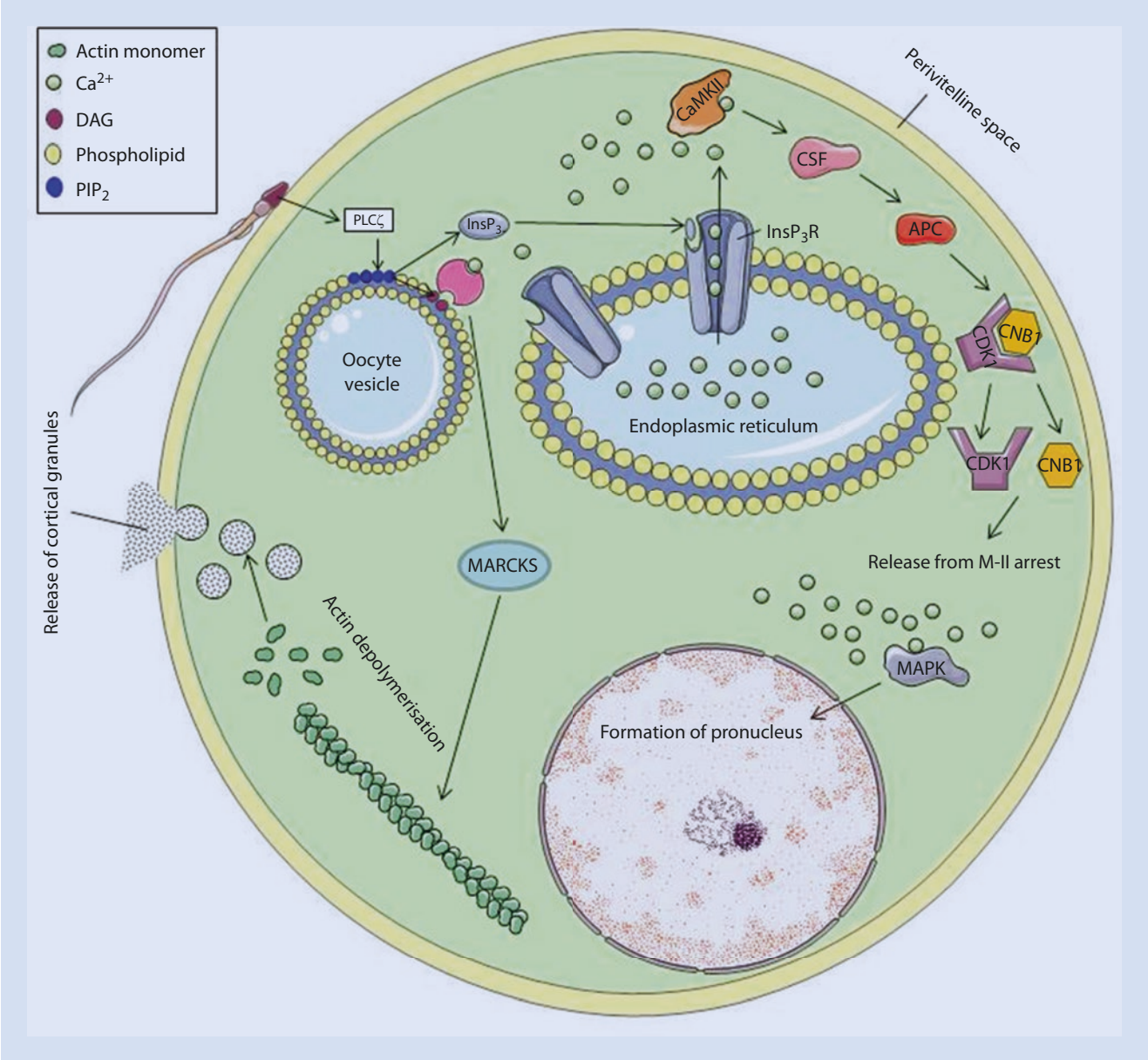

Fig. 34.2 Oocyte activation triggered by PLCζ, a sperm-specific protein factor. (Reproduced with permission from Yeste et al. [105])

shortly after fertilization [47, 107, 108]. Ca^{2+} binds calmodulin (CaM), a ubiquitous Ca^{2+} sensor protein, which activates calcium/calmodulin-dependent protein kinase II (CaMKII) [109–111] (Fig. 34.2). Subsequently, CaMKII phosphorylates F-box protein 43 (FBXO43), also known as early mitotic inhibitor 2 (EMI2), which is an anaphase-promoting complex inhibitor which maintains the levels of maturation-promoting factor (MPF) and thus M-II arrest. In brief, MPF is a heterodimer of cyclin-dependent kinase 1 (CDK1), previously known as cell division cycle 2 (CDC2), and cyclin B1 (CCNB1). FBXO43 inhibits the anaphase-promoting complex/cyclosome (APC/C) and blocks CCNB1 degradation [112–115]. The phosphorylation of FBXO43 by CaMKII leads to its degradation via ubiquitin-mediated proteolysis, in a process that involves Polo-like kinase 1 (PLK1), and results in MPF degeneration which allows the oocyte to be alleviated from M-II arrest. Upon completion of meiosis II, the pronuclei is formed in a process that is also reliant upon Ca^{2+} levels and involves the MAPK pathway [116] (Fig. 34.2).

In turn, Ca^{2+} oscillations and cytoplasm movements terminate as a consequence of a negative feedback regulation mechanism, as sustained levels of IP_3 lead to the downregulation of IP_3Rs [117–121]. Indeed, high cytoplasmic Ca^{2+} levels inhibit IP_3R via CaM/CaMKII, and since there is no Ca^{2+} release after IP_3R inhibition, Ca^{2+} levels in the cytoplasm are reduced, and a new Ca^{2+} oscillation may occur [122]. It is also worth mentioning that not only does IP_3R play a crucial role during oocyte activation events but also during oocyte maturation and early embryo development [123, 124].

It has been suggested that DAG remains attached to the oocyte vesicles, but this hypothesis has yet to be proven. Diacylglycerol should interact with protein kinase C (PKC), a Ser/Thr protein kinase, via its C1 domain. At least in the case of conventional PKC isoforms, PKC is also regulated by Ca^{2+} through the C2 domain [125]. An activated PKC phosphorylates multiple substrates, via C3 and C4 domains, in a manner which remains to be identified [126]. Among the known substrates, PKCα phosphorylates myristoylated alanine-rich C-kinase substrate (MARCKS) proteins, which are involved in the regulation of actin network and thus in the prevention of polyspermy [127–129]. PKCs are also converted into catalytic subunits (PKC-M), which subsequently distribute across the ooplasm and phosphorylate cytoskeleton proteins and other substrates [130, 131]. Finally, while it is yet to be proven whether any Ca^{2+} pump involved in producing Ca^{2+} oscillations in mammalian oocytes is phosphorylated by PKC, one should note that voltage-gated calcium (Cav2.2) channels in *Xenopus* oocytes are regulated via their phosphorylation by PKCs [132].

The distribution of PKC isoforms has been reported to be related with oocyte activation, as specific isoforms play precise roles during oocyte maturation and activation upon fertilization. Indeed, PKCδ and PKCθ are also involved in oocyte maturation, specifically in the meiotic resumption of germinal vesicle oocytes [133]. PKCα is uniformly distributed before fertilization and translocates to the oocyte cortex thereafter, showing kinetics similar to the Ca^{2+} oscillations induced by sperm [134]. In addition, PKCα is implied in the generation of the second polar body and the extrusion of cortical granules [127, 135]. Finally, it is worth mentioning that PKCβ1 is also involved in oocyte activation, as it is translocated from the cytosol to the oocyte plasma membrane following fertilization [136].

34.4 The Significance of Calcium (Ca^{2+}) Oscillation Patterns in the Oocyte and Their Role in Controlling the Molecular Mechanisms of Oocyte Activation

As previously described, oocyte activation events are mainly driven by Ca^{2+} oscillations, and an increase in Ca^{2+} levels within the ooplasm is critical in allowing M-II resumption. It is thus crucial to comprehend how relevant the proper management of Ca^{2+} homeostasis is for oocyte maturation and activation, as anomalies in this homeostatic mechanism may affect the oocyte's ability to be activated [105]. Ca^{2+} oscillations are evoked by the SOAF and exhibit complex temporal and spatial properties which begin shortly after fertilization and persist beyond the completion of meiosis II [43, 57, 107, 137, 138]. This indicates the existence of a very precise regulatory mechanism which controls how intracellular stores are filled, emptied, and refilled. Related to this, it is worth noting that the oocyte takes up extracellular Ca^{2+}, which is crucial for filling the intracellular stores which are then emptied by the action of the SOAF. If fertilization takes place in an extracellular environment devoid of Ca^{2+}, Ca^{2+} oscillations evoked by the SOAF terminate earlier [139].

The major factors responsible for Ca^{2+} homeostasis are proteins which constitute the store-operated Ca^{2+} channels (SOCE) which manage Ca^{2+} entry into intracellular stores [140, 141]. The main components of this system, which are gated channels activated when Ca^{2+} levels in the intracellular stores are depleted and allow Ca^{2+} influx from the extracellular space to the cytosol and endoplasmic reticulum, are stromal interaction molecule-1 (STIM1), Ca^{2+} release-activated Ca^{2+} channel protein 1 (ORAI1), sarco/endoplasmic reticulum Ca^{2+}ATPase (SERCA), and other unidentified membrane channels (Fig. 34.3) [140–143].

The particular role of these proteins has also been shown in mammalian oocytes [144], as STIM1 and ORAI1 are involved in Ca^{2+} influx to the oocyte and subsequent Ca^{2+}-filling of internal stores during oocyte maturation [145, 146]. STIM1 is a single-pass, type I transmembrane protein which acts as a sensor for Ca^{2+} levels within the ER and features an EF hand domain and a sterile alpha motif domain in the lumen of the ER [147–149]. The sensing ability of STIM1 is related to ORAI, a Ca^{2+} channel, which is activated by STIM1 and allows extracellular Ca^{2+} to enter [150–152]. The interaction of STIM1 with ORAI1 occurs via direct protein-protein interaction involving the ER (STIM1) and the plasma membrane (ORAI1; [146, 153, 154]). In addition to ORAI1, there are also plasma membrane Ca^{2+} ATPases (PMCAs) which pump Ca^{2+} from the cytosol into the extracellular milieu [155]. This helps to increase the cytosolic levels of Ca^{2+}, which are used to refill the ER, as detailed below.

In combination with STIM1, ORAI1, and PMCAs, sarco/endoplasmic reticulum Ca^{2+} ATPases (SERCAs), which reside in the membrane of the ER, are also implicated in Ca^{2+} homeostasis as they are responsible for the active transport of Ca^{2+} from the cytosol to the lumen of the ER [156, 157] (Fig. 34.3). The main role of these ATPases is to continuously fill the ER with Ca^{2+}. In effect, without the pumping activity of SERCAs, a second, new Ca^{2+} oscillation cannot be generated after emptying the lumen of the ER for the first time following the interaction of IP_3 with its receptor [141]. The most important SERCA isoform is SERCA2b which is organized into clusters when the oocytes reach the M-II stage and maintain this organization until the pronuclear stage is reached, which concurs with the duration and pattern of Ca^{2+} oscillations [139].

Yet, it should be mentioned that mitochondria are also important for modulation of Ca^{2+} signaling [156, 158]. It has been reported that oocytes with altered mitochondrial function and reduced ATP production show an abnormal Ca^{2+} oscillation pattern [159]. Furthermore, oocytes may enter an apoptotic mitochondria-mediated caspase pathway when there is excessive Ca^{2+} oscillatory activity [160]. Therefore, the involvement of mitochondria in the dynamics of Ca^{2+} homeostasis should also be contemplated when trying to understand the events underlying oocyte activation.

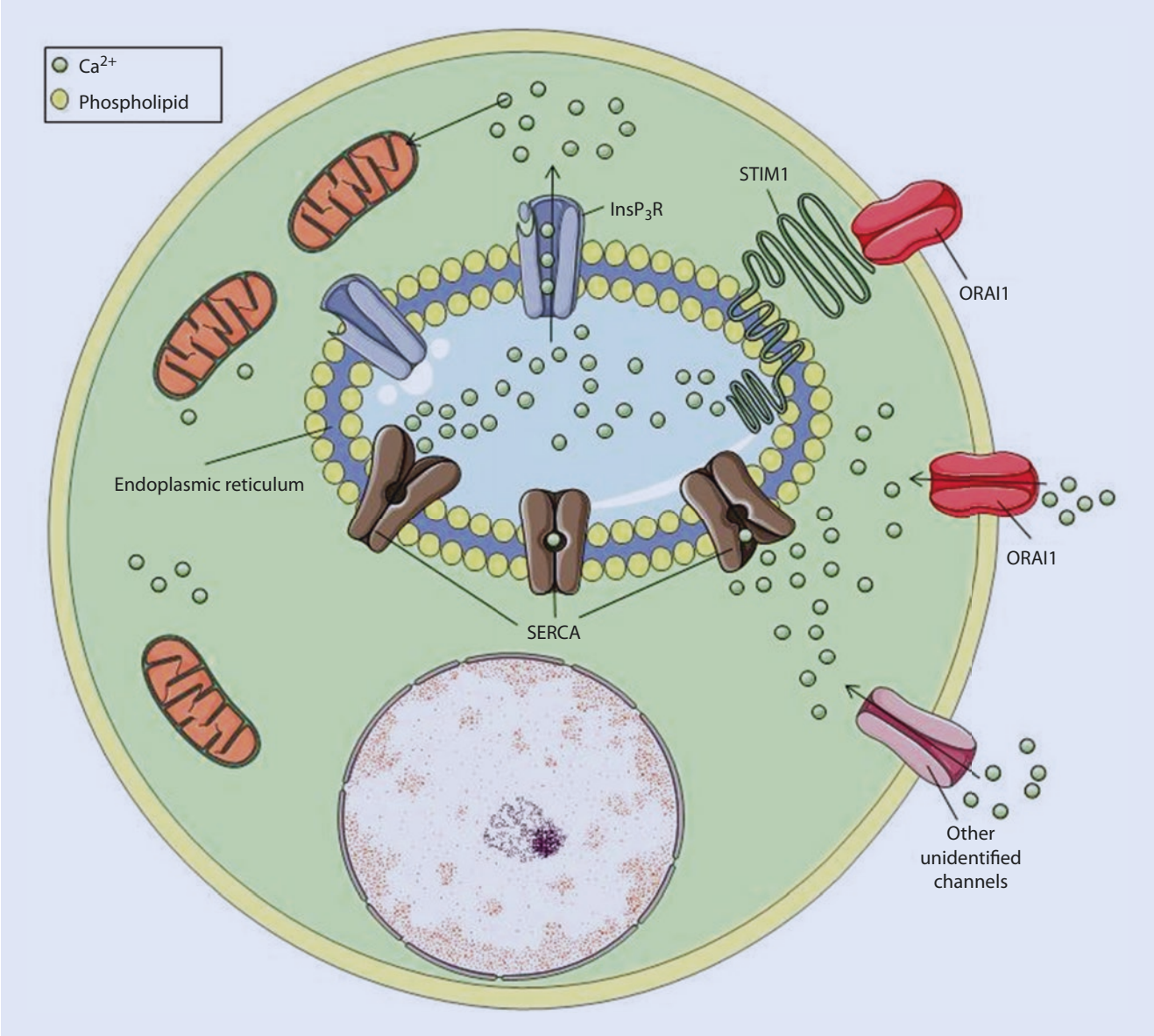

Fig. 34.3 Oocyte mechanisms involved in the regulation of Ca^{2+}. It is important to note the relevance of SOCE (SERCA, ORAI1) for Ca^{2+} homeostasis, as well as the role of mitochondria. (Reproduced with permission from Yeste et al. [105]). Abbreviations: SOCE, store-operated calcium entry; SERCA, sarco/endoplasmic reticulum calcium ATPase; ORAI1, calcium release-activated calcium channel protein 1

34.5 Oocyte Activation Deficiency in the Context of Human Infertility

Having outlined what oocyte activation is and the main events involved, we should now focus upon the impact of oocyte activation deficiency (OAD) on human infertility. Infertility is defined as the inability of a sexually active couple to achieve pregnancy after 1 year of unprotected vaginal intercourse [161]. With an incidence between 10% and 16% of couples [162–166], infertility is now considered as a disease by the World Health Organization [167].

Regarding the origins of infertility, a male factor is predominantly involved in between 20% and 30% of cases, a female factor is thought to underlie between 20% and 35% of cases, and both male and female factors are implicated in 25–40% of cases (reviewed by Yeste et al. [105]). In the remaining 10–25% of cases, the precise cause of infertility remains unexplained. The strategies to treat infertility depend upon the origin, but in some cases, assisted reproductive technology (ART), which includes in vitro fertilization (IVF), intracytoplasmic injection (ICSI), and embryo transfer, may be suitable to rectify at least some of these cases (reviewed in Yeste et al. [105]).

One specific cause of infertility is oocyte activation deficiency (OAD), in which the oocyte fails to activate following sperm-oocyte fusion. Intracytoplasmic sperm injection, whereby a single sperm is directly injected into the oocyte, is a suitable alternative to rescue fertility when IVF fails [12]. However, total fertilization failure can still occur in 1–5% of ICSI cycles (reviewed in Yeste et al. [105]). While the causes

that may underlie such failure are of a broad nature and can include a variety of technical factors (e.g., incorrect injection) and sperm (poor nuclear chromatin condensation) and oocyte (spindle) defects [168, 169], OAD is considered to be the principal cause of total fertilization failure and low fertilization rates following ICSI, accounting for 40% of such cases [170, 171].

Although, as previously stated, abnormalities and deficiencies in PLCζ may explain some cases of OAD, the causes of oocyte activation failure are not necessarily restricted to sperm defects but may also involve the oocyte, since oocyte quality plays a pivotal role in fertilization success (reviewed by Yeste et al. [105]). Indeed, as OAD and total fertilization failure may also occur when spermatozoa present normal PLCζ patterns [68, 172], sperm factors other than PLCζ and oocyte proteins involved in the critical downstream signaling events triggered by the SOAF could also underlie OAD, as suggested in previous investigations [173–176]. It has already been emphasized that oocyte quality, morphology, and age together with specific oocyte proteins may be responsible for OAD [177–181]. Therefore, further research on the potential contribution of the oocyte in OAD is much warranted [95, 105]. However, ethical issues and restrictions on the availability of healthy human oocytes donated for research make conducting studies with female gametes far more difficult than with sperm.

34

34.6 Artificial Oocyte Activation

Over recent years, artificial oocyte activation (AOA) has represented the only treatment option to rescue cases of total fertilization failure that are related to OAD. Artificial oocyte activation is defined as the use of any laboratory method which helps overcome OAD and thus facilitates oocyte release from M-II arrest [174]. This procedure is advised when fertilization rates are below 30% following conventional ICSI and allows the rescue of cycles in which severe male factor infertility and deficient oocyte maturation are involved [174, 175, 182–184]. In spite of this, AOA may not be a suitable strategy when the suspected factor underlying OAD is related to the oocyte [181]. It is also worth mentioning that the pattern of Ca^{2+} oscillations triggered by AOA differs greatly from that observed following normal fertilization, as a single Ca^{2+} transient rather than a series of physiological Ca^{2+} oscillations is usually observed [169] (Fig. 34.4). Finally, one should also note that the use of AOA is not only restricted to rescuing fertility, but this technology may also be utilized for nuclear transfer and parthenogenesis [186].

34.6.1 Methods to Activate the Oocyte Artificially

The suite of methods for activating the oocyte artificially are classified into three main groups: mechanical, electrical, and chemical [171, 187].

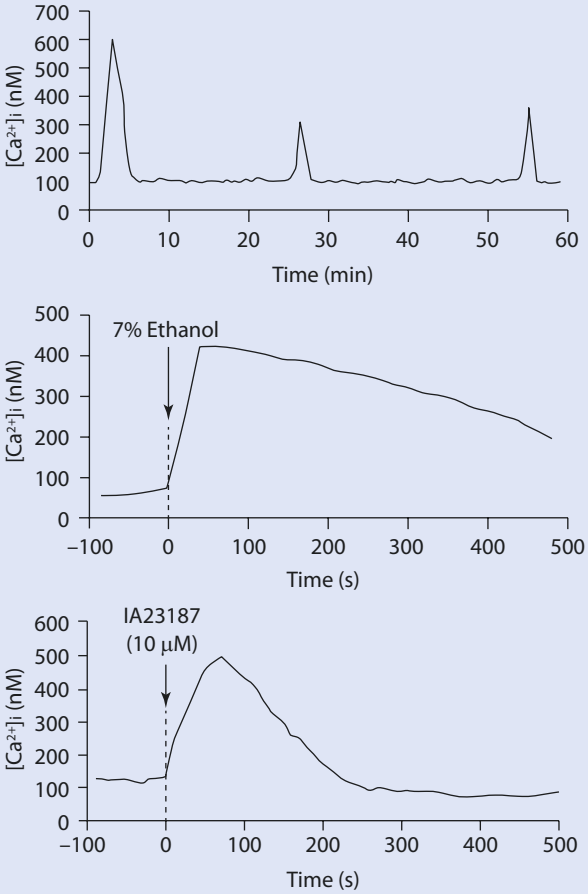

Fig. 34.4 Calcium oscillations in bovine oocytes after in vitro fertilization **a** and in response to artificial oocyte activators (**b**, ethanol; **c**, calcium ionophore A23187). Note that whereas the characteristic pattern of Ca^{2+} oscillations is present after IVF, a single Ca^{2+} spike is observed when chemical activators are used. (Reproduced with permission from Nakada and Mizuno [185])

Mechanical oocyte activation consists of vigorous aspiration of the oocyte peripheral cytoplasm and further deposition of such aspirated cytoplasm and the spermatozoon in the center of the oocyte during ICSI. This peripheral cytoplasm has mitochondria with high inner membrane potential and ATP content which, when deposited in the center of the oocyte, are thought to increase energy supply in the site of pronuclear formation and facilitate oocyte activation [188]. Another way to mechanically activate the oocyte is the disruption of its plasma membrane immediately before ICSI using a microneedle, as this allows maintaining Ca^{2+} influx [187]. However, these mechanical methods are not common in clinical practice, as they are difficult to standardize, are invasive, and entail a higher risk of damaging the oocyte [189].

Electrical oocyte activation stimulates oocytes electrically using electrodes and an electric chamber. In brief, oocytes are subjected to a single, pulsed-direct current waveform. This electric stimulus opens pores in the plasma membrane, allows Ca^{2+} influx from the surrounding medium, and ultimately increases the intracellular Ca^{2+} levels [169, 190].

Artificial oocyte activation via the use of chemicals is currently the most common clinical procedure, as success rates are higher than those obtained with either mechanical or electrical stimulation [191]. In the case of chemical stimulation, oocytes are, following ICSI, transferred to a medium which contains an AOA. It is worth mentioning that most agents cause a single, prolonged Ca^{2+} transient in the oocyte (Fig. 34.4), whereas others cause multiple oscillations.

Calcium ionophores, such as ionomycin and calcium ionophore A23187, and inhibitors of kinase activity and protein synthesis, such as 6-dimethylaminopurine and puromycin, cause a single Ca^{2+} transient [175, 192–198]. Specifically, calcium ionophores are lipid-soluble molecules, which bind to ions and work as ion carriers, thus allowing the transport of such ions across the plasma membrane, which is impermeable to them otherwise. Therefore, the exposition of oocytes to calcium ionophores increases permeability of the plasma membrane to Ca^{2+}, which allows extracellular Ca^{2+} to flow into the oocyte from the surrounding medium and increases intracellular Ca^{2+} levels in a single transient manner. This single Ca^{2+} transient triggers downstream events which are driven by Ca^{2+}/calmodulin and CaMKII [183]. Calmodulin kinase II may remain active, although to a lesser extent (50–80% kinase activity), when partially autonomous (Ca^{2+} independent) following dissociation from Ca^{2+}/calmodulin. This kinase activity lasts until phosphatases dephosphorylate CaMKII and override its autophosphorylation activity [199].

Chemical agents which cause multiple transients include strontium chloride, phorbol esters, thimerosal, and anhydrous alcohol [200–203]. Although strontium chloride is the most used and is effective in mice, its use in humans is still under debate [171, 204, 205]. In spite of this, it has been indicated that strontium chloride may be appropriate in those clinical cases in which calcium ionophore A23187 is proven to be ineffective [201]. While the mechanism by which strontium chloride induces Ca^{2+} oscillations is not fully understood, it appears to bind to and activates a Ca^{2+}-binding site on the IP_3R [206, 207]. On the other hand, it is worth indicating that the use of anhydrous alcohol as an agent for AOA has been shown to improve blastocyst development in vitrified GV and M-I oocytes following in vitro maturation [203].

There are only a limited number of studies comparing the effectiveness of these three AOA methods. Vanden Meerschaut et al. [208] evaluated embryo development, pregnancy, birth, and fertility of pups using a mouse model for globozoospermia (wobbler mouse) exhibiting oocyte activation deficiency. These authors reported that electrical activation and chemical activation with strontium chloride were more efficient in rescuing fertility and supporting embryo development than ionomycin. In spite of this, ionophores are the most used chemical agents in humans and show reasonable results [208]. Another study with parthenogenetic mouse and human embryos observed that blastocyst development at day 6 was higher following electrical stimulation than in response to chemical activation with either ionomycin or strontium chloride [186].

34.6.2 Artificial Oocyte Activation in Clinical Practice

Available data on the use of AOA agents are encouraging, but this is yet to be considered as an established treatment [171]. Unfortunately, a systematic review and meta-analysis which compared conventional ICSI with ICSI+AOA concluded that, in general, fertilization rates are higher following ICSI-AOA than following ICSI. Indeed, the studies reviewed were so different that no evidence on the efficacy or safety of ICSI-AOA could be concluded [209]. The specific concerns with regard to AOA safety will be discussed later in this chapter.

Ionomycin and calcium ionophore A23187 are the most common AOA agents used in clinical practice. The first case of AOA using calcium ionophore A23187 was reported in 1997 [210]. Fertilization rates of artificially activated oocytes with calcium ionophore A23187 range from 25% to 48%, and live birth rates are around 28% [211]; nonetheless, differences have been observed between studies. For example, Aydinuraz et al. [212] did not find a clear effect of AOA with calcium ionophore A23187 upon cleavage rates but did observe that the number of high-quality embryos was increased in the group of artificially activated oocytes.

Artificial oocyte activation with calcium ionophore A23187 has also been found to be efficient when using in vitro matured oocytes [213]. Yet, not only is this procedure effective with ejaculated sperm of good quality but also with ejaculated oligoasthenoteratozoospermic spermatozoa and even with testicle-extracted spermatozoa [214, 215]. Interestingly, AOA with calcium ionophore A23187 was found to rescue a case of male infertility associated with Kartagener syndrome [216]. The man suffering from this syndrome, which is related to primary cilia dyskinesis, presented severe oligoasthenoteratozoospermia with sperm unable to respond to theophylline, a motility activator. In this case report, treating oocytes with calcium ionophore A23187 was shown to allow fertilization and blastocyst formation and resulted in a successful pregnancy and the birth of two healthy twins. In contrast, conventional ICSI produced no fertilized oocytes [216].

Still related to calcium ionophore A23187, it is worth mentioning that a recent study has found that the combination of calcium ionophore A23187 with recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) had synergistic effects and increased cleavage rates, embryo development, and the number of euploid blastocysts [217].

34.6.3 Is AOA Safe for Child Health?

Artificial oocyte activation represents a relatively new therapeutic strategy which can rescue some cases of total fertilization failure. However, there are concerns with regard to

the safety of the procedures involved. Despite Ca^{2+} being a ubiquitous secondary messenger involved in several vital processes, artificial oocyte activators induce an increase in the intracellular Ca^{2+} levels which is not regulated by the endogenous molecular machinery inside the oocyte. In effect, artificial activators and artificially generated Ca^{2+} oscillations could impact upon gene expression and epigenetic regulation [183, 218, 219]. For this reason, it is of great concern that we still know so little about the specific downstream events which could be affected by unregulated changes in Ca^{2+} concentration in oocytes and early embryos; these concerns have fueled significant debate on the safety of this ART procedure [171, 187]. As a consequence, AOA is still considered as an experimental procedure and should only be applied under specific circumstances, when no alternative solution is possible [183].

More importantly, the existing literature has not yet fully addressed whether the use of AOA leads to any specific adverse effects. Although there are some reports indicating an impact upon gene expression following ICSI combined with AOA, the number of studies is limited, and the sample size involved is low. Moreover, there are very few follow-up investigations on the children born following AOA. In this context, it is worth emphasizing that natural fertilization has a series of control mechanisms which are bypassed by ART in differing extents depending on the method used (i.e., IVF, ICSI, ICSI-AOA). While some biological mechanisms of sperm selection are still present in IVF, such mechanisms are bypassed by ICSI. The extent of this bypass is still higher when AOA is used, as the Ca^{2+}-driven events within the oocyte are not triggered by the sperm presence but rather through a chemical agent which does not interact naturally with the oocyte's internal machinery governing Ca^{2+} homeostasis. In fact, the risk of abortion, miscarriage, and some syndromes associated with imprinting errors is higher with conventional ICSI than with natural conception [220, 221]. It is therefore highly prudent to increase the number of studies addressing these effects and risks in the case of AOA, and the development of other more endogenous, therapeutic strategies would be of significant interest [222]. Although, at present, AOA is the only suitable strategy, the use of a recombinant human PLCζ isoform would inevitably represent a far safer strategy for rescuing oocyte activation deficiency, as discussed in the next section.

The limited amount of data presently available indicate that the use of calcium ionophores appears to be safe and does not cause any detrimental impact upon child health [174, 184, 223, 224]. For example, a study using calcium ionophore A23187 reported a case of major malformation in a baby born using AOA with this chemical agent [216]. However, and according to the authors of this case report, the risk of congenital malformations increases when IVF and ICSI are utilized, so that it is not possible to ascertain whether this case could be explained by the use of ART in general or whether other factors were involved [216]. However, another study observed activation by calcium ionophore A23187 and in almost 80% of cases found no detrimental effects of artificial activation upon chromosome segregation errors during meiotic division, after genotyping and maternal haplotyping these oocytes [225]. Moreover, a retrospective study using data compiled from 2006 to 2014 compared pregnancy and birth defects between ICSI (595 cycles) and ICSI-AOA oocytes (83 cycles) and found no significant differences in the time of delivery, percentage of twin pregnancies, birth defects, type of defects observed (chromosomal and structural abnormalities), and malformations [226].

In the case of ionomycin, comparisons of babies born through ICSI and ICSI, combined with AOA and ionomycin, showed no effects upon chromosomal abnormalities, intrauterine fetal death, preterm delivery, birth weight, growth rate, hospitalization in neonatal intensive care units, abnormal behavior according to age, and the physical and mental health of children born [220].

Finally, although no detrimental effects of strontium chloride have been observed in terms of physical and mental development of children from birth to 60 months [201, 204, 205], a study conducted by Bridges et al. [227] did indentify effects upon gene expression. Briefly, this research compared gene expression in mouse blastocysts produced by IVF, conventional ICSI, and ICSI+AOA with strontium chloride. When conventional ICSI and IVF blastocysts were compared, the expression of 197 genes, involved in cell differentiation, development of the organs and nervous system, cell-cell signaling, transduction pathways, and several metabolic processes, was found to differ. The expression of 132 genes was also found to differ between IVF and ICSI-AOA blastocysts. When comparing ICSI and ICSI-AOA blastocysts, the expression of 65 genes, involved in different developmental, metabolic, and response processes, also differed [227]. These data support the idea that AOA is still an experimental treatment and should only be advised in particular cases until further knowledge about the exact mechanisms by which AOA agents exert their effects is available.

34.6.4 Is AOA Always Useful for Rescuing Fertility?

It is important to consider that artificial oocyte activation may not be effective enough when causes other than oocyte activation deficiency are evident. This is the case of severe globozoospermia, involving mutations/deletions of the *DPY19L2* gene. In such cases, AOA is able to rescue oocyte activation, but due to the fact that sperm chromatin is poorly compacted, presents an aberrant protamine content, and DNA is fragmented, the resultant rates of preimplantation embryo development are low [82].

On the other hand, while research with calcium ionophore A23187 found no effect in terms of sperm source (i.e., ejaculated or epididymal), Borges et al. [228] observed different outcomes when using ionomycin and oocytes from women of different ages. Indeed, the outcomes of AOA with ionomycin using ejaculated, epididymal, and testicular spermatozoa did not differ when the oocytes fertilized originated from women below 36 years of age. However, when AOA with ionomycin was performed with oocytes from women

above this age threshold, the proportion of high-quality embryos was higher with the use of ejaculated and epididymal spermatozoa, and implantation rates were higher with the use of ejaculated spermatozoa [228].

Finally, we should mention that apart from utilizing AOA for severe cases of sperm and oocyte deficiencies, another strategy has proposed the use of this technique with calcium ionophore A23187 to increase fertilization and pregnancy rates of women with a diminished ovarian reserve and whose partners present normal sperm parameters. However, such a strategy has been revealed to be ineffective, as it does not significantly increase fertilization or pregnancy rates [229].

34.6.5 Could Recombinant PLCζ Represent an Effective Agent to Rescue OAD?

This chapter has emphasized the importance of Ca^{2+} homeostasis and that Ca^{2+} oscillations evoked by sperm upon gamete fusion present a characteristic pattern. However, and as previously discussed, Ca^{2+} oscillations triggered following IVF and ICSI differ from those generated when AOA is used (Fig. 34.4) [169]. This has led the scientific community to express concerns about the potential side effects of AOA [169, 230]. One possible strategy to rescue oocyte fertility would be the use of a recombinant PLCζ protein, as Ca^{2+} oscillations evoked by this recombinant sperm-specific protein resemble those observed following conventional fertilization and IVF. This approach has attracted the attention of researchers working in this field. Yoon et al. [48] demonstrated that cRNA encoding PLCζ could rescue fertility in an infertile patient whose sperm were devoid of PLCζ and did not activate the oocyte following ICSI. In addition, Kashir et al. [75] were the first to report the production of a recombinant human PLCζ protein, which was found to be able to elicit the characteristic Ca^{2+} oscillations triggered by sperm after normal fertilization [75]. The species specificity of recombinant PLCζ forms was further demonstrated by Nomikos et al. [45] who showed that the ability of recombinant human PLCζ to initiate embryo development in mice was lower than in humans. Finally, in a study conducted with mouse oocytes, Sanusi et al. [46] compared AOA with recombinant PLCζ, calcium ionophore, and strontium chloride and found that PLCζ gave higher rates of embryo development than calcium ionophore. A more detailed review of the potential use of recombinant PLCζ protein as a more endogenous alternative to AOA agents is beyond the scope of this chapter, but we refer the reader to Amdani et al. [231] for further information. At the time of writing, progress on the clinical translation of recombinant PLCζ protein remains ongoing and is some way from being introduced into clinical practice.

34.7 Conclusions

This chapter has defined the process of oocyte activation and has described the main events that take place during this crucial phenomenon. As oocyte activation is triggered by sperm upon gamete fusion, we have mainly summarized the most relevant research findings pertaining to the identity of the sperm-borne oocyte activation factor (SOAF). Although other candidates have been proposed over the last two decades, mounting evidence now indicates that a sperm-specific phospholipase C, PLCζ, is the primary SOAF candidate. We have also reviewed the molecular pathway triggered by PLCζ and emphasized the crucial role of Ca^{2+} oscillations and Ca^{2+} homeostasis within the oocyte. Finally, we have described how some cases of human infertility relate to OAD and how AOA is increasingly being considered as a strategy to rescue fertility in couples suffering from OAD. Finally, due to concerns being raised about the clinical use of AOA, we have described the limitations of this technique and suggested other potential and safer alternatives.

Conflict of interest The authors declare that there is no conflict of interest with regard to the content of this chapter.

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Review Questions

1. What is oocyte activation?
2. Which are the molecular mechanisms underlying that process and what is still unknown?
3. Which is the role of sperm in oocyte activation upon sperm-oocyte membrane fusion?
4. Is there any relationship between sperm devoid of PLCζ and oocyte activation deficiency and total fertilization failure?
5. Are there any means to overcome oocyte activation? Are they safe?

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Micromanipulators and Micromanipulation

Contents

Hydraulic Manipulators for ICSI

Hubert Joris

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Learning Objectives

- Overview of micromanipulators and integration to microscopes
- Different components of a hydraulic micromanipulation system
- Alignment of microtools for ICSI
- Maintenance of hydraulic micromanipulation systems

Micromanipulation of cells started more than a century ago. Already in 1859, a description of some kind of microdissector had been made. The first studies on combined micromanipulation of oocytes and embryos appeared around 100 years later. Research on early events of fertilization was the main reason for these studies. In these 100 years between the first description of micromanipulation and the use of this technique for the study of fertilization events, the technological evolution changed our society completely, also improving conditions for such research.

Medical treatments did benefit a lot from scientific progress. Helping infertile couples to conceive was also part of this progress. The birth of Louise Brown in 1978 has been a milestone that changed the world of assisted reproductive technology (ART). The use of IVF evolved rapidly from treatment of female infertility to treatment of male infertility. However, it was obvious that the number, motility, and morphology of the spermatozoa present in the ejaculate largely affected the success rates of IVF. The idea to assist fertilization by bringing sperm cells closer to the egg vestments was already studied in animals in the early 1960s. These techniques have been evaluated and used in humans to assist the fertilization process. This evolution is the subject of other chapters in this book. However, for these studies, the instruments developed over the years to study and manipulate different types of cells showed to be very valuable to manipulate gametes.

Different types of manipulators have been developed and used. The main purpose of this equipment is to bring movements from a macroscale to a microscale without affecting the viability of the material worked with. Transfer of the movement from a macroscale to a microscale can be done in different ways. This can be done mechanically, hydraulically, and electronically. Mechanical and electronic manipulators and their use in ART is the subject of different chapters.

At UZ Brussel, research on assisted fertilization started in the late 1980s aiming for the establishment of a clinical-assisted fertilization program. Assisted fertilization by subzonal insemination (SUZI) in the mouse was successful and demonstrated a correlation between the level of acrosome-reacted sperm cells and fertilization after insertion of a single spermatozoon under the zona pellucida [1]. Micromanipulation of gametes was initially performed with mechanical manipulators (Leica, Wetzlar, Germany). After careful evaluation of different possibilities, they were replaced by a combination of electrical and hydraulic manipulators (Narishige, Japan). This micromanipulation system was used at the start of the clinical-assisted fertilization program. The initial clinical experience by SUZI [2] was soon followed by the first report on intracytoplasmic sperm injection (ICSI) pregnancies [3]. The combination of electrical and hydraulic manipulators showed to be a very successful combination. The aim of this chapter is to describe more in detail the function and characteristics of this micromanipulation system for the ICSI procedure.

35.1 Mounting Micromanipulation Systems on Microscopes

Manipulation of cells smaller than what can be observed by the human eye requires the use of microscopes magnifying the cells to a level allowing proper observation of their characteristics. Manipulation of these cells requires a system that allows a firm, steady movement that successfully executes the required action without damaging the biological material submitted to the manipulations. To visualize human oocytes to a level where morphological details can be observed, a magnification of 200× to 400× is very common. Sperm cells are much smaller, and details cannot easily be observed at that magnification. Movement of tools small enough to manipulate these cells without vibration requires a very steady and firm system. Independent of the way micromanipulators are driven, they need to be mounted so that vibration is minimal. Depending on the microscope available and the choice of the micromanipulation system, manipulators are built on the microscope or on the microscope table or placed next to the microscope. These mounting systems allow a very steady positioning of the manipulators but do not necessarily avoid vibration. Several possibilities of equipment absorbing vibration are available and can be installed at different levels. This can be under the table, under the table surface, or directly under the microscope. However, such antivibration systems have a limited capacity, and certain vibrations will not be absorbed. Avoiding vibration is an important aspect to consider when establishing a new laboratory or when installing a new micromanipulation system.

35.2 Micromanipulation System

Micromanipulators transfer movements from the macrolevel to the microlevel scale. This involves a transition from a movement at the centimeter scale to a movement at the millimeter or micrometer scale. Movements executed with the control units are transferred to the drive units on which tools or tool holders are mounted. For IVF purposes, cells are manipulated by glass tools mounted in a holder, which is then fixed on the drive unit of the manipulator. These tools are usually made from borosilicate glass capillaries. Using a pipette puller, microforge, and if required a grinder allows production of microtools with specific characteristics.

For ICSI purposes, a holding and injection pipette is required. Commercially available products are used mostly

nowadays. The micropipettes are fixed in the holder, and the holder is mounted on the universal joint of the drive unit. Before the actual manipulation can start, the tools have to be positioned and aligned, allowing easy manipulation procedures. The position of the tools before starting the alignment can be considered the *starting position*. Practically, it is important that the tools can easily return to their starting position, for example when dishes are replaced. For this purpose, course manipulators are used. These allow easy movements at the centimeter scale that can be driven mechanically or electrically. Details of the alignment procedure using this manipulator system are described later.

Probably, the most commonly used set of micromanipulators for ICSI is from Narishige. These micromanipulators were initially developed for research purposes and are still commonly used in different areas of research [4]. Different types of manipulators have different ranges in their movements and are used for different applications. As such a system had proven to be successful for ICSI [5, 6], it has been introduced in many IVF clinics all over the world. For use in IVF, a combination of coarse manipulators allowing movements in the centimeter range and fine manipulators with movements at the micrometer scale allowing easy movement covering the microscope field at a magnification of 200× or 400× is adequate (Fig. 35.1). The movement covering the view field at a magnification of 400× is a movement of around 500 µm.

The coarse manipulators allow movements at the centimeter scale. These movements can be driven mechanically or electrically. A possible advantage of a mechanical system is presence of less electrical cables in the laboratory, but it requires significant movements with the arms each time these manipulators are used. The major advantage of the electrical coarse control manipulator is the presence of the joystick next to the microscope. This allows performance of all different manipulation steps within reach and without losing visual control over the biological material visible under the microscope.

Fig. 35.1 Example of setup for ICSI with electrical coarse manipulators and hydraulic manipulators for fine movements. (Courtesy of Nikon, Melville, NY, USA)

35.3 Hydraulic Micromanipulators

Besides the coarse manipulator, a manipulator for fine movements is required. This manipulator is the most important one. The combination presented here uses a hydraulic micromanipulator for fine movements. Similar to the electrical manipulator, a major advantage of such a manipulator is that the joystick can be placed close to the microscope and next to other joystick(s) or injector(s) avoiding excessive movements during the manipulation procedures. The hydraulic manipulator consists of two main parts, namely, the control unit and the drive unit. The control unit (Fig. 35.2) is placed close to the microscope. Its base consists of a magnetic stand, and it is usually fixed on a metal plate by the magnetic switch. In this way, it remains steadily in the same position and allows easy maneuvering of the joystick. The parts of the control unit used during the manipulations are the joystick, allowing movements in two dimensions, and the three rotating knobs, each allowing movement in one dimension. One of the rotating knobs is positioned at the end of the joystick. The two other rotating knobs are positioned above the joystick. The three rotating knobs can be rotated over a certain range. At the 0 level, there is no hydraulic pressure from the system on the moving parts. Rotation of each of the knobs creates movement in one axis. The length of the movement caused by one complete rotation of the knob depends on the characteristics of the manipulator used. Manipulations can be performed using the rotating knobs individually. However, the major advantage of the manipulator used here is that movements in three dimensions can be controlled simultaneously by using the joystick and the rotating knob at the end of the joystick simultaneously. The amplitude of the joystick movement during the manipulations can be regulated using the *movement ratio adjustment ring*. The sensitivity of the movement can be regulated with the *tension adjustment ring*. This three-dimensional movement is an important feature during the different steps of the manipulation procedures performed on human oocytes, embryos, or sperm cells.

Fig. 35.2 Example of control unit of hydraulic manipulator with hanging joystick. (Courtesy of Nikon, Melville, NY, USA)

The joystick can be in a hanging or upright position. In the early 1990s, the hanging joystick could be used only in combination with Nikon microscopes. Later on, this changed. Hanging joysticks are now available for different brands of microscopes. Compared to the joystick in an upright position, the hanging joystick gives important advantages when it comes to ergonomics. Since the embryologist may spend hours at the microscope, this is an important aspect. A hanging joystick allows maintaining the hands at the same level when switching between the electrical course manipulator, the hydraulic manipulator, and the injector and allows supporting with the wrist on the table while working with the hydraulic manipulator.

The second part of the hydraulic manipulator is the drive unit (Fig. 35.3). This is mounted on the coarse manipulator and connected with the control unit by three tubings filled with oil. The tool holder can be fixed in the universal joint that is mounted in the drive unit. As such, movement of the drive unit is transferred directly to the microtool. The movement of the knobs or the joystick creates pressure, and this pressure is transferred by the oil to the moving parts of the drive unit. The pressure created results in an immediate response and causes movement of the drive unit that is in proportion to the amplitude and speed executed on the joystick and/or rotating knobs. It is this movement that is the most crucial in the manipulation process. This direct and proportional transfer of movement performed at the control unit and delivered to the drive unit gives a perception of direct control. This is a very important feeling giving confidence to the operator.

Adjusting the amplitude by the ratio adjustment ring to cover the complete view field at the largest magnification ICSI that is performed at the beginning with the joystick in the neutral position creates a very comfortable working area and allows continuous visual control of the tools during the manipulation steps.

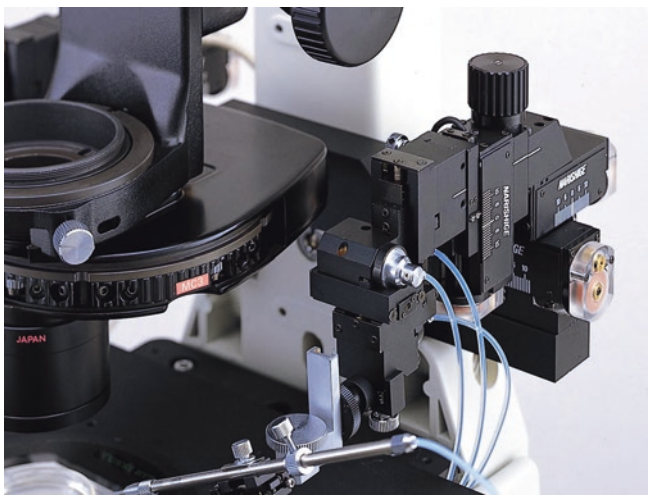

Fig. 35.3 Example of drive unit of hydraulic manipulator (connected to control unit by tubing) mounted on electrical coarse control manipulator and universal joint for mounting of the tool holder. (Courtesy of Nikon, Melville, NY, USA)

35.4 Alignment of Microtools

Proper positioning and alignment of the microtools before starting the injection procedure is crucial for successful ICSI. Although ICSI is considered a routine procedure, it still occurs that micromanipulators are not used optimally and tools are positioned in a way that can affect results. Like certain knowledge about the use and adjustment of CO_2 incubators is required for control of proper functioning during culture, certain minimal knowledge about the characteristics and possibilities of the manipulators is necessary.

Considering the possibilities this equipment has, there is not just one-way positioning and alignment that is performed correctly. The procedure described hereafter is a procedure used during the many years worked in the IVF lab of UZ Brussel.

Before starting the procedure, it is safe to check that both the coarse and the fine manipulator are more or less in a central position. This avoids interruption during the ICSI procedure because one of the manipulators reached the limit of its movement possibility in one of the different directions. The procedure then starts with mounting ICSI and holding pipette in the tool holder and fixing the tool holder on the universal joint. Holding and injection pipette are placed on the left hand side and right hand side, respectively. The tubings connecting the tool holder with the injector as well as the ones between the control unit and the drive unit of the hydraulic manipulator should be free and without any excessive bending that can possibly interfere with correct transfer of the command to the manipulator or microtool.

The design of the universal joint allows positioning of the tool holder in numerous different positions (Fig. 35.3). The older version of the universal joint had two changeable parts, while more recent types have three different parts that can be moved or rotated. As this material was not designed for IVF purposes only, movement in a large spectrum was necessary. However, if ICSI is the only procedure performed, limited adjustments are required once a more or less optimal position is established. Although produced under strictly controlled conditions, individual microtools may vary slightly in certain characteristics that require different settings of the universal joint.

Once the holders are fixed in the universal joint, the tip of the microtool can be moved in the light beam of the microscope. The moving and rotating parts of the universal joint may be used for this if required. Depending on the type of microscope used, it may be useful to do this initial positioning (starting position) high enough above the microscope stage, allowing dishes to be removed and placed under the microtools easily. More recent models of microscopes have a tilting arm on which the manipulators are mounted. By tilting the arm, the complete manipulation system is lifted. This facilitates movements with dishes when microtools are mounted.

For all manipulations related to IVF applications, microtools with similar basic characteristics are used, meaning that capillaries with a diameter of around 1 mm are

formed into tools with different specifications. In the vast majority of the cases, tools are shaped so that the end part of the glass needle is bent to an angle between 20° and 40°. This allows easy positioning of the microtools with the final bent part being positioned almost horizontally once fixed on the universal joint.

Once the two needles are brought in the light beam of the microscope, positioning and alignment controlled via the image in the microscope start. Easiest is to start these steps using the objective with the lowest magnification (often 4×). Without changing the view plane, using the coarse manipulators, the microtools are lowered until they reach the plane where they can be seen clearly (Fig. 35.4a). If required, by rotation of the tool holder, the microtools are rotated until the bent part is positioned in a line going from the 3 o'clock to the 9 o'clock position. Both needles are moved to the center of the image. An ideal alignment brings both microtools in one straight line from the 3 o'clock to the 9 o'clock position (Fig. 35.4b). Further detailed positioning is performed after changing the objective stepwise to 10×, 20×, and finally 40×. Whether the bent part is placed horizontal or deviates from the optimal position (Fig. 35.5) may not be that clear in this image for less experienced operators. When placing the microtools in a medium droplet of a dish, this can be seen more easily. This positioning is performed by using the coarse manipulators for the large movements and the hydraulic manipulators for the fine movements. When using only the rotation knobs of the hydraulic manipulator during these steps, the joystick remains in the neutral position, allowing optimal movement ratio when working at larger magnification.

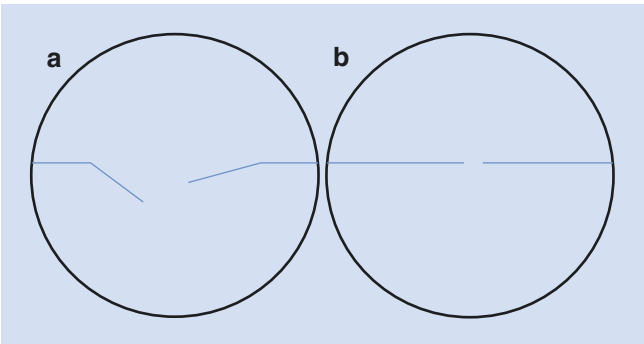

Fig. 35.4 Top view of microtools before initial alignment **a** and after initial alignment **b**. Holding pipette on the left hand side, injection pipette on the right hand side

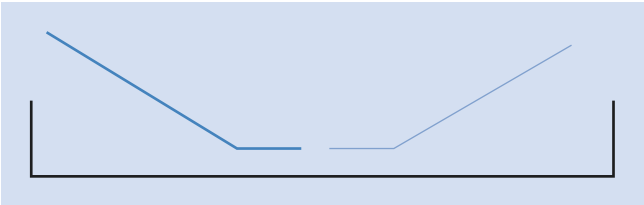

Fig. 35.5 Correct position of microtools at the start of the ICSI procedure. The bent tip is positioned horizontally

The way the drive unit and universal joint are constructed and mounted allows positioning of the tool holder in almost unlimited ways. As mentioned before, slight variation in the angle of individual microtools may require adjustment of the angle for an optimal injection procedure. It is at this stage that modification of the position of the tool holder can easily be performed. After having lifted the tool holders to the starting position, one can easily modify the angle of the tool holder and bring the bent part in a more favorable position. Correct adjustment to place the tools in an optimal position will allow an easy procedure and plays a role in the success rates obtained. In the early days of ICSI, these changes were made by using the different possibilities of the universal joint and required some extra manipulation. Nowadays, improvements in the universal joint allow fine changes in certain axes by simply turning a small screw on the universal joint without additional manipulation of the tool holder (Fig. 35.6).

Ideally, the bent part of the microtools should be in an almost completely horizontal position after alignment (Fig. 35.5). Incorrect positioning of the microtools will affect the manipulation process and can result in positions as shown in Fig. 35.7. Touching the bottom of the dish with the tip of the pipette is not possible when the needle is placed as shown in Fig. 35.7, position 2. This results in difficulties fixing the oocyte in the case of the holding pipette or does not allow aspiration of a sperm cell from the bottom of the dish nor to immobilize a sperm cell with the tip of the needle in case the injection pipette is positioned in this way. In cases where the tip is placed as shown in Fig. 35.7, position 3, one may not have sufficient support of the holding pipette during the penetration of the ICSI pipette in case it is the holding pipette that is placed like this, or one may not make a straightforward movement when injecting a sperm cell into the oocyte. If a tip is aligned as shown in position 3, only the very end of the tip is in focus, while the rest of the pipette cannot be seen sharp in

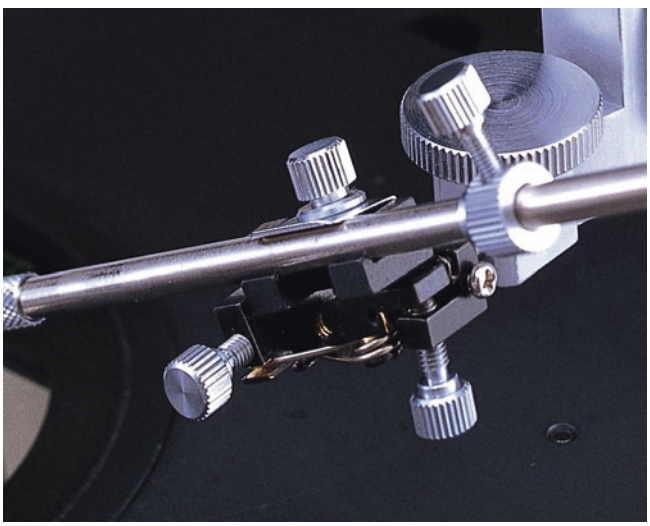

Fig. 35.6 Example of a more recent type of universal joint where changes in position can be made by simple rotation of screws with mounted tool holder. (Courtesy of Nikon, Melville, NY, USA)

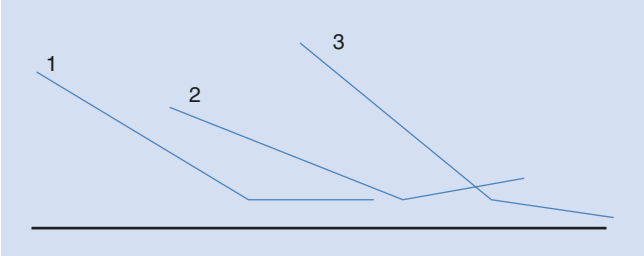

Fig. 35.7 Different positions of microtools: (1) correct position; (2) tip of the pipette cannot reach the bottom of the dish; (3) only the very end of the tip touches the bottom of the dish

the same view plane. When working at the microscope, we have a two-dimensional image while working in three dimensions. Except for the changes in sharpness of the tools when looking at a certain view plane, deviation from the horizontal position of microtools is not visible. However, minimal trauma to the oocyte is created only if the tip of the injection pipette is placed horizontally. It may not be easy to quantify the effect of suboptimal positioning on the microtools. However, like for all other aspects of IVF, attention to detail makes the difference. This is not different when it comes to ICSI. Once microtools are positioned correctly, the ICSI procedure can start.

As for any type of equipment used in the lab, the manipulation system requires maintenance. Maintenance of the moving parts using certain types of grease (performed by technicians trained by the company) results in continuous normal functioning of the manipulators. As the movement from the joystick to the drive unit is a hydraulic system, this part of the system may need to be changed, but only after a very long time of use. It is the author's experience that it took more than 10 years of intensive daily use until the pressure of the hydraulic system became insufficient, and reparation was required in one of the hydraulic manipulators. One can easily significantly increase the life span of the manipulator by turning the three rotating knobs to the 0 (zero) position at the end of each working day. This releases the pressure in the system.

In summary, hydraulic manipulators are usually only part of a complete micromanipulation system. The combination of mechanical or electrical coarse manipulators and hydraulic manipulators for fine movements is used extensively for IVF applications. The combination of the rotating joystick and the rotation knob that are driven together by hand allows easy three-dimensional movement. The hydraulic pressure results in a movement of the microtool that is in proportion to the amplitude and speed executed on the joystick. This gives a sensation of direct control over the manipulation. Furthermore, the way the manipulators and tool holder are designed offers a large scale of possibilities and allows easy adjustment to the type of tool used and kind of manipulation performed.

Acknowledgment The permission of Nikon to use pictures showing different manipulators is greatly acknowledged.

Review Questions

1. Describe the major components of a hydraulic micromanipulator.
2. What is the basic principle of a hydraulic micromanipulator?
3. Describe the two types of joysticks and the functions of the joystick.
4. Describe the steps involved in the alignment of micropipettes in a hydraulic micromanipulator.

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Research Instruments Micromanipulation Systems

Steven Fleming and Catherine Pretty

Z. P. Nagy et al. (eds.), *In Vitro Fertilization*, https://doi.org/10.1007/978-3-319-43011-9_36

Learning Objectives

- To provide an overview of the company and the manufacturing principles underlying Research Instruments equipment
- To describe the basic steps required for the successful installation and operation of Research Instruments micromanipulation systems
- To discuss the various applications of Research Instruments micromanipulation systems within the context of assisted reproduction technology
- To recommend various procedures for overcoming difficulties or problems that may be encountered when using Research Instruments micromanipulation equipment

36.1 The Ethos of Research Instruments

36.1.1 Company History

In response to the requirement for integrated circuit testing by microelectronics companies such as GEC, Research Instruments (RI) was established by Mike Lee and Vince Grispo in 1962. Following an approach by Dr. Simon Fishel during the 1980s, RI adapted their product output in order to meet the needs of assisted reproduction practitioners attempting to perfect new techniques for male factor infertility, such as partial zona dissection (PZD) and subzonal insemination (SUZI).

The company launched the first CE marked micropipette in 1998. The Saturn laser system was designed by David Lansdowne in 1999 and, following the addition of software and firmware capability, became the world's first computer-controlled laser for assisted reproduction technology (ART). The company was bought from Mike Lee, Vince Grispo and Ann Lee by Bill Brown, Justin Retallack and David Lansdowne in 2000 and went on to become an innovative leader within the field of development and manufacture of equipment for ART. In 2016, RI was incorporated into the CooperSurgical group of companies, joining forces with ORIGIO, The Pipette Company (TPC), K-Systems, Genesis Genetics, Recombine, Reprogenetics and Wallace, market leaders in ART.

36.1.2 Research and Development

The earliest purely mechanical micromanipulators manufactured by RI, such as the TCV500 that was released in 1964, featured individual levers with three axes of movement and movement reduction from 100:1 to 500:1. The TLO500 was introduced in the 1980s, incorporating flexural hinges in order to improve stability and reliability. The subsequent development of the classic TDU500 enabled mounting onto an inverted microscope and extremely ergonomic manipulation. Accessory instrumentation, including the 'Sonic Sword', was also introduced at this time to facilitate the penetration of the *zona pellucida* (ZP), primarily for the SUZI technique.

In the early 1990s, following the successful application of intracytoplasmic sperm injection (ICSI) to the human [1], RI modified their micromanipulators to introduce a number of useful features. The first of these allowed rapid raising and lowering of micromanipulators, avoiding accidental damage to the micropipettes and, therefore, was referred to as a 'Home Function'. An innovative feature, laser-guided setting-up device (LASU) launched in 1994, facilitated correct alignment of micropipettes. Other features added during the 1990s were tool holder angle adjustment indicators for more accurate alignment of a range of micropipettes with various known degrees of bend between the tip and the shank. Precisely controlled heated plates were integrated into the mechanical stage in 1999, culminating in a major modification of the TDU500, known as the 'Integra'. RI also introduced semicircular handrests to further improve ergonomics.

36.2 Equipment Installation and Set-Up

36.2.1 Integra Design Features

The current version of the Integra is known as the Integra 3™ (Fig. 36.1).

The Integra 3™ incorporates a mechanical stage, TDU3 micromanipulators, shortcut buttons to link with RI Viewer software, touchscreen four-channel independent control of

Fig. 36.1 The Integra 3™

heated plates, one-touch 'home' function, one-touch micropipette angle adjustment (Fig. 36.2) and a unique touch-screen display with stopwatch and counter (Fig. 36.3). On the Integra 3™, the standard mechanical stages of the 'big four' microscope manufacturers have been replaced by a custom-designed, built-in XY mechanical stage. Each turn of the stage control moves the stage plate 28 mm in either the X or Y plane, with 40 mm full travel. Because the mechanical stage is supplied fitted to the Integra 3™, no set-up is required. The independent four-channel temperature control system is accurately calibrated to within 0.1 °C of the desired set point. One of these channels controls the temperature of the central metal insert in the microscope stage. As an optional upgrade, RI will supply a central glass heated stage. Even better temperature control is provided by the Thermosafe™ warm air system. One channel controls the entire working stage plate. This has been designed for temperature maintenance of microinjection dishes additional to that being worked on at any given time, e.g., in those circumstances where it is necessary to have a testicular sperm preparation in one dish and oocytes ready to be injected in another. The fourth channel can be used to control the temperature of an external heated plate or the heated stage of an adjacent stereo dissecting microscope. The four channels are controlled via a touch-screen panel built into the Integra 3™ (Fig. 36.3). As the heated stages are already installed when supplied, no set-up other than the adjustment of the temperature set points is necessary.

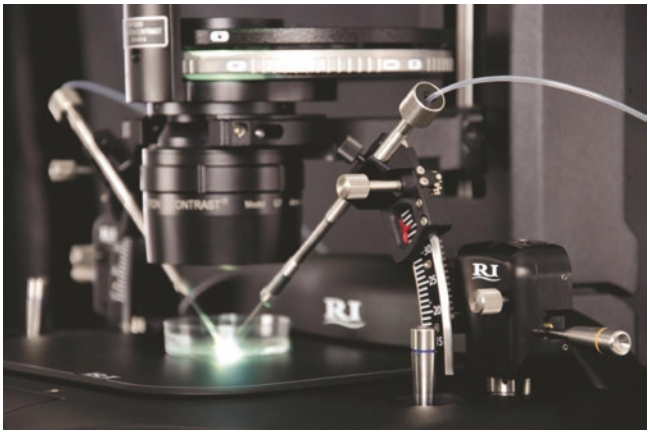

Fig. 36.2 One-touch micropipette angle adjustment

Fig. 36.3 Touch-screen digital display

Compared with that of the previous model, the Integra Ti, the heated stage area of the Integra 3™ has been increased by 40% and is enhanced by an integral monitored and alarmed heating system called Thermosafe™. This system creates a uniform flow of precisely warmed air towards the bottom of the micromanipulation dish, thereby circumventing the known cooling effect of the underlying objective lens which otherwise acts as a heat sink, drawing heat away from the heated stage. Also, the Integra 3™ now benefits from a motion sensor LED light situated below the heated stage which illuminates the objective lenses, making it much easier to visualize the objective lens required for the purpose.

Consistent with the original company policy, the TDU3 micromanipulators remain purely mechanical and are directly controlled, the joysticks providing proportional movement. This policy is based on the principles that direct proportional movement provides greater control and that simple mechanical components are ready to use, are more reliable and are less likely to require routine servicing and maintenance.

36.2.2 Integration with Inverted Microscopes

Installation of the Integra 3™ is extremely simple, quick and straightforward as the system comes largely preassembled in a purpose-built dispatch case. However, it is still necessary to exercise some care when removing the Integra from its dispatch case in order to avoid potential damage to the micromanipulator mechanism from any inadvertent severe shock. An installation manual is available from RI in Adobe Acrobat format. The Integra 3™ can be adapted to a range of inverted microscopes supplied by the 'big four' manufacturers, Leica, Nikon, Olympus and Zeiss using just four screws (Fig. 36.4).

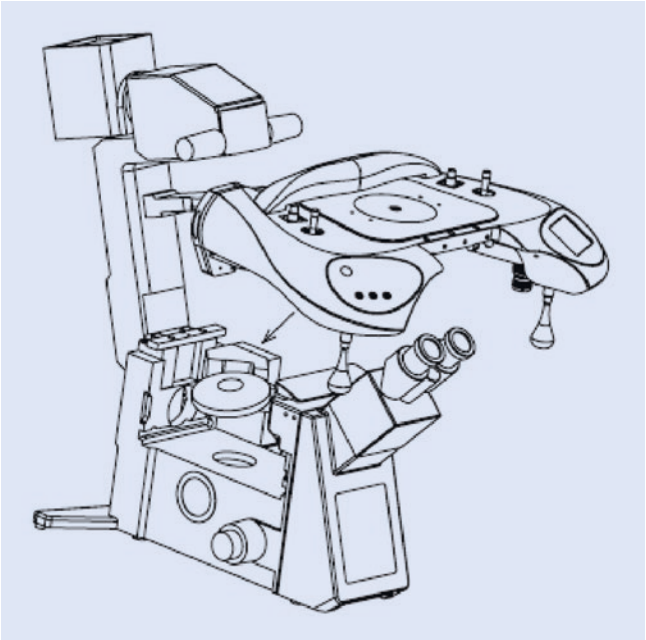

Fig. 36.4 Installation diagram for the Integra 3™ to the Olympus IX73

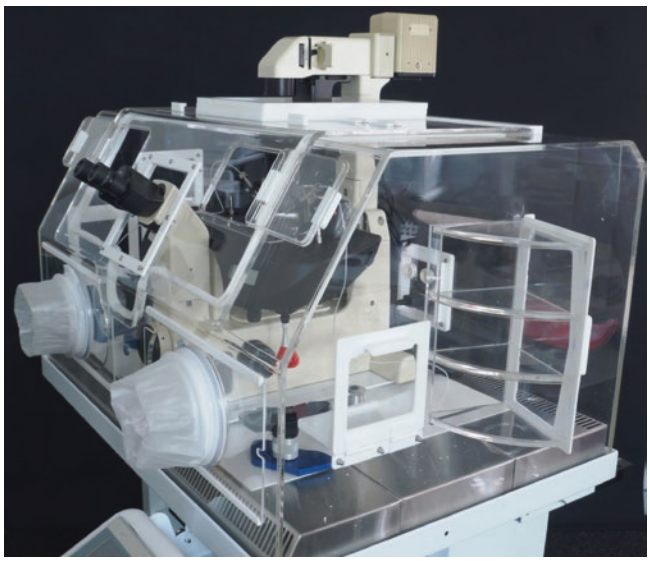

Fig. 36.5 Cell-Tek 3000 IV enclosed chamber for an inverted microscope

36.2.3 Integration with Workstations and Chambers

Since micromanipulation is usually conducted in closed culture under oil using media incorporating buffers (e.g., HEPES and MOPS) that maintain stable pH under atmospheric conditions, it may be performed out in the open on the lab bench. However, there are practitioners who prefer the aseptic conditions offered by a laminar flow cabinet (LFC) or environmentally controlled conditions provided by an enclosed microscope chamber (Fig. 36.5). Such chambers allow micromanipulation to be performed with the assurance that temperature, pH and humidity will remain at optimal levels for the entire duration of the procedure. Some chambers also incorporate inbuilt ultraviolet photocatalytic modules that reduce volatile organic compounds to low parts per billion levels.

Most horizontal and vertical LFCs provide sufficient space within which to house an inverted microscope fitted with a micromanipulation rig. However, some will need to be specially adapted for this purpose so that the eyepieces of the microscope project through an aperture in the front window. The microscope will impede the laminar flow to some extent but, nevertheless, an LFC will provide more aseptic conditions than the open lab bench. To minimize vibration during micromanipulation, it is advisable to use a low vibration LFC and to place an anti-vibration plate between the base of the microscope and the floor of the LFC. Similar anti-vibration measures have to be taken if opting to house a micromanipulation system within a microscope chamber, such as the inbuilt anti-vibration pad that accommodates any microscope configuration within the chamber available from Tek-Event.

36.2.4 Micromanipulators

The micromanipulators on the Integra 3™ incorporate both fine and coarse controls in one compact unit. Both fine and coarse control levers should be set to their vertical positions before making further adjustments. In common with the classic TDU500 micromanipulator, the joysticks of the TDU3 also extend downwards, but now from within the microscope stage of the Integra (Figs. 36.1 and 36.3). Rotation of the fine control knob actuates up to 5 mm of movement within the z-axis so, in order to avoid running out of travel mid-procedure, it is recommended to set its travel of movement at the midpoint prior to use. As a guide to determining the midpoint of micromanipulator travel, green electronic height indicators (Fig. 36.3) enable precise setting of the manipulators on each side and provide an audible warning alarm just before the end of the travel has been reached.

36.2.5 Tool Holders and Micropipettes

RI's PL3 tool holders are supplied with the Integra 3™ and are calibrated and actuated using a single screw to enable accurate adjustment to a range of micropipette bend angles, from 16° to 43° (Fig. 36.2). This is important to ensure an optimal angle at which a micropipette is employed respective to the procedure being undertaken so as to achieve effective manipulation while minimizing shear stress. A unique feature of the PL3 tool holder is that the tip of the micropipette does not move when the angle is changed, allowing alterations of the pipette angle to be made 'on the fly'. Once the angle of the tool holder has been set to correspond to the bend angle of the micropipette to be used, RI's MPH microtool holders can be clipped into place onto the PL3 tool

holders (Fig. 36.2). Once fitted to each other, vertical, axial and rotational movement of the micropipette is possible, allowing a rapid set-up.

36.2.6 Setting Up and Aligning Micropipettes

When adjusted using an additional objective lens and spacer (supplied by RI), micropipettes can be aligned 14 mm above the microscope stage, minimizing the likelihood of damaging them. A one-off initial set-up of the tool holder is achieved as follows: A scratch or mark is made on the inner surface of a Petri dish and the dish is placed onto the microscope stage of the Integra 3™. Using the 4× objective lens, the scratch is brought into focus to establish the set focal length for tool holder set-up, and is not adjusted further. A holding micropipette is fitted to the microtool holder and, with the vertical lever of the tool holder in the fully raised position, the objective lens is changed over to the RI 4× objective lens with spacer which allows the micropipette to be brought into focus in this raised position. Check that the micromanipulator is set to the middle of its vertical travel and that both fine and coarse control levers are positioned in their vertical axes. Drive the MPH microtool holder forward until the tip of the micropipette is directly above the objective lens. Rotate the microtool holder until the micropipette vertically bisects the objective lightpath, as viewed with the naked eye, and then tighten the bottom securing screw of the PL3 tool holder (Fig. 36.6). This adjustment effectively sets the detent position of the microtool holder to its central position.

For routine micropipette set-up, next use the axial drive mechanism of the PL30 tool holder to position the micropipette within the centre of the field of view and rotate it axially using the rotating wheel at the distal end of the MPH microtool holder to obtain a perfectly vertical orientation of the micropipette (Fig. 36.2) – precise alignment is best achieved with the aid of the inverted microscope, viewing the micropipette using the 4× or 10× objective lens and using the fine control lever to bring its tip into focus. If necessary, move the 4× objective lens back into place and fully lower the vertical lever on the PL3 tool holder – the scratch or mark on the Petri dish should still be in focus. Because the micropipette is positioned slightly above the surface of the Petri dish, it will now appear slightly out of focus. If desired, the distance between the tip of the micropipette and the surface of the microinjection dish can be minimized by increasing the distance travelled when lowering the vertical lever on the PL3 tool holder. This adjustment is achieved using the lowered position adjustment screw located on the top of the PL3 tool holder (Fig. 36.6). These steps are repeated when setting up the injection, zona drilling or biopsy micropipette on the opposite side of the micromanipulation rig. As an optional extra for those wishing to biopsy embryos for the purpose of

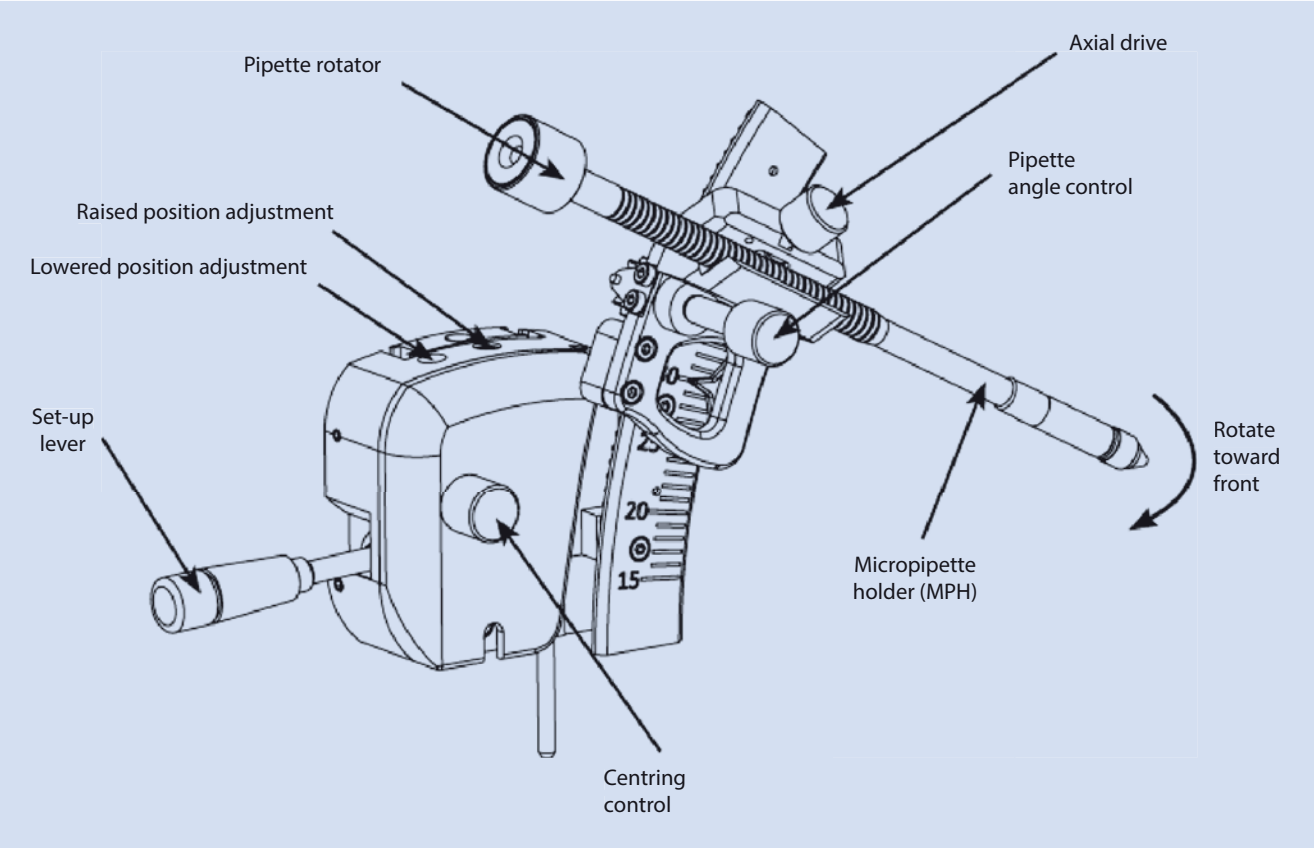

Fig. 36.6 PL3 tool holder

pre-implantation genetic testing (PGT), a double tool holder that enables independent movement of two micropipettes on the one tool holder is available from RI. However, with the advent of laser systems such as RI's Saturn 5 Active™ laser, most practitioners have now moved away from the use of double tool holders.

36.2.7 Microinjectors

The RI screw-actuated syringes (SAS) supplied as standard with the Integra 3™ are air-assisted microinjectors that are sometimes referred to as 'mushrooms' due to their inverted mushroom-shaped design (Fig. 36.7). By virtue of their heavy circular base, they are very stable, yet they occupy a relatively small footprint. With a capacity of 2 ml, they can generate high aspiration and pressure. The SAS air injector also benefits from incorporating a pressure release button situated on top of the screw control that enables rapid equilibration of internal and external pressure (Fig. 36.7). This feature is particularly useful for stabilizing capillary flow and for rapid cessation of aspiration pressure applied to rupture the oolemma during ICSI. An extra-smooth chrome special edition of the SAS microinjector is available from RI as an optional extra. For those who prefer to use an oil microinjector, RI will supply a micrometer-actuated sealed oil syringe (SOS) mounted on a sturdy, non-slip base.

The SAS microinjectors are supplied with hard polythene tubing for connecting them to the MPH microtool holders. It is a simple procedure to attach the tubing at one end to the metal nozzle underneath the SAS microinjector and, at the other end, to the proximal end of the MPH microtool holder. Once the tubing has been connected and a micropipette fitted to the MPH microtool holder, the micropipettes must be primed with media and the SAS microinjectors equilibrated prior to use. For the holding micropipette, this procedure simply requires the micropipette to be lowered into a drop of micromanipulation medium and the SAS to be rotated slowly anticlockwise until media rushes in, at which point the pressure release button can be pressed down in order to equilibrate the pressure inside and outside of the micropipette, thereby halting the influx of the medium. Priming and equilibrating an injection micropipette is a slightly longer process. Firstly, the SAS needs to be rotated fully clockwise to its lowest position and the pressure release button pressed. Then, the micropipette can be lowered into a drop of micromanipulation medium or a 7–10% polyvinylpyrrolidone (PVP) solution and the SAS rotated several turns anticlockwise, approximately 50% of its travel upwards. It may then be necessary to wait for up to 5 min in order for the media to be aspirated into the micropipette until it almost reaches the unpulled shank. At this point, the pressure release button can be pressed down to ensure the equilibration of the pressure, preventing unwanted drift of media up or down the micropipette. For those using older SAS models that do not incorporate a pressure relief button, it is necessary to disconnect and reconnect the tubing at the SAS microinjector in order to achieve equilibration during priming of micropipettes.

36.3 Clinical Applications

36.3.1 Zona Drilling and Assisted Hatching

Zona drilling is the technique of creating a hole in the ZP surrounding the oocyte or preimplantation embryo, originally described in 1986 [2]. Although less frequently used these days, the most widely used application of zona drilling in assisted reproduction, termed 'assisted hatching', is based on the assumption that the blastocyst will 'hatch' more readily from the ZP if previously breached by drilling a hole or cutting a slit in it. A variety of factors, including excessive thickness of the ZP and zona hardening following embryo culture, were originally proposed as one cause of recurrent implantation failure, due to the inability of blastocysts to 'hatch' [3]. Various means have been used to achieve 'assisted hatching' including the use of acids, enzymes and lasers to dissolve the ZP, no single method proving universally better

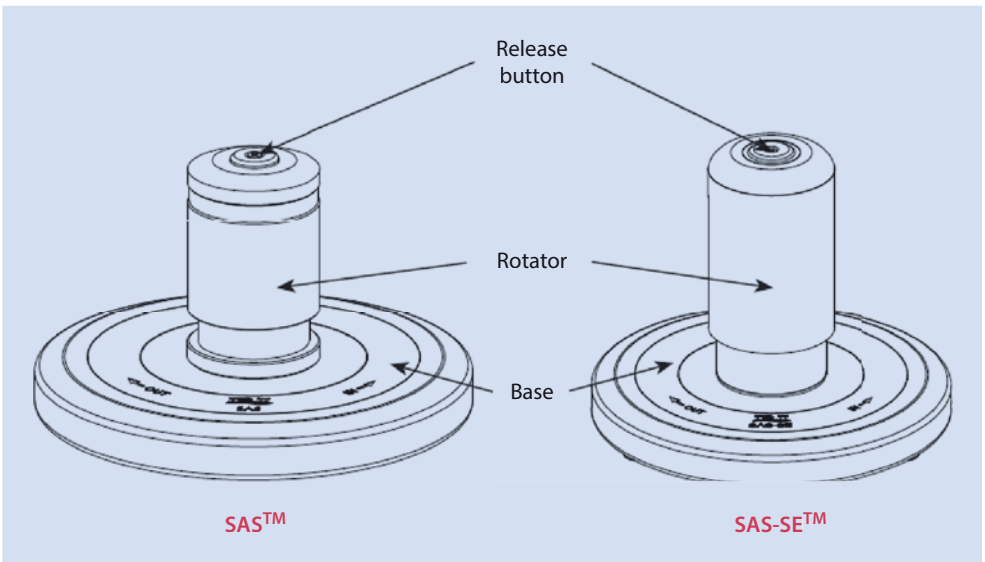

Fig. 36.7 SAS™ and SAS-SE™ air injector with equilibration button

than another [4]. It can be achieved by chemical means through controlled and directed application of acidified Tyrode's solution (pH 2.3–2.5) or pronase, using a drilling micropipette attached to a microinjector. For the flow of acidified or enzymatic media to be precisely controlled, a drilling micropipette should be fire-polished to an internal diameter of 5–10 µm, which is much smaller than the internal diameter of a holding micropipette. Contact and non-contact lasers, operating in either the ultraviolet or infrared spectrum, may also be applied to the ZP at a single point or at several adjacent points, depending on the length of hole desired. Lasers dissolve the ZP by generating heat, so non-contact lasers, especially those operating within the infrared spectrum, are generally considered safer for use with human oocytes and embryos. Zona drilling has also been used for other applications such as fragment removal from early cleavage-stage embryos and embryo biopsy for PGT.

36.3.2 Partial Zona Dissection

One of the earliest applications of zona 'drilling' in the human, termed 'zona tearing' or PZD, was to create a conduit for access to the oocyte by spermatozoa deemed incapable of binding to and penetrating the ZP [5, 6]. In this approach, micropipettes are used to physically cut a slitlike hole in the ZP while holding the oocyte firmly onto a holding micropipette.

36.3.3 Subzonal Insemination

At around the same time that PZD was developed, the PZD technique was being combined with the use of large microinjection pipettes in order to introduce spermatozoa directly into the subzonal perivitelline space [7, 8]. Originally termed 'microinjection sperm transfer' (MIST), the technique later became known as 'subzonal insemination' (SUZI). Once the manufacture of fine, sharp microinjection pipettes had been perfected, PZD became redundant for the purposes of SUZI.

36.3.4 Intracytoplasmic Sperm Injection

The technique known as intracytoplasmic sperm injection (ICSI) represents the ultimate evolution of experimental methods to alleviate male factor infertility, such as PZD and SUZI [1, 9]. The vastly superior efficiency of ICSI in achieving monospermic fertilization of the oocyte resulted in its rapid replacement of the SUZI technique. Successful application of ICSI depends on an appreciation that it must mimic the latter stages of fertilization that occur in vivo [10]. As with gamete fusion, the sperm plasmalemma and oolemma have to be temporarily breached. Hence, the microinjection pipette should be set up at such an angle that the sperm plasmalemma can be ruptured using the tip of the pipette, illustrated by a permanent kink in the sperm tail. Likewise, the tip of the microinjection pipette must be sharp enough that the oolemma ruptures when aspirated onto it using a microinjector, as evident by sudden free-flow of ooplasm into the microinjection pipette. Modifications of ICSI, such as laser-assisted ICSI [11], could feasibly result in improvements to the technique, though their relative benefits and risks need to be considered further.

36.3.5 Oocyte, Embryo and Blastocyst Biopsy

Following zona drilling, cells can be removed from the oocyte, early cleavage-stage embryo and blastocyst as biopsy material for the purposes of PGT, this technique having been pioneered soon after that of 'assisted hatching' [12]. Since the majority of aneuploidies occur during oocyte maturation [13], the first polar body (PB) represents a useful source of material for PGT, so methods have been developed to perfect PB biopsy. However, since postzygotic aneuploidy is also possible, although less common, blastomere biopsy of early cleavage embryos at the eight-cell stage has tended to be the approach preferred by those testing for sex-linked disease and other genetic mutations. More recently, partly because of the possibility of misdiagnosis due to mosaicism in the early cleavage-stage embryo, trophectoderm biopsy of the blastocyst has assumed greater importance.

Biopsy micropipettes should have an internal diameter of 40–50 µm. If a laser is not available for zona drilling, it will be necessary to fit drilling and biopsy micropipettes to a double tool holder that allows rapid interchange between the two during a biopsy procedure. For the purposes of blastomere biopsy, the optimal size hole to be drilled in the ZP should be only just large enough to allow a biopsy micropipette to enter the perivitelline space. One or two blastomeres may be removed from a seven- or eight-cell embryo, two blastomeres providing greater control for the potential for a misdiagnosis. With trophectoderm biopsy of the blastocyst, a commonly applied method is to allow a small portion of trophectoderm to herniate from the ZP following previous zona drilling on day 3 of development, and then to use a laser to help separate the extruded trophoblast.

36.3.6 Cytoplasmic and Mitochondrial Transfer

Some patients, particularly those of advanced maternal age, may remain infertile due to poor oocyte quality, yet may prefer to have their own biological children rather than resort to oocyte donation. Hence, cytoplasmic transfer was introduced as a potential means of rectifying perceived deficiencies or defects within the oocytes of patients experiencing repeated implantation failure [14]. Cytoplasmic transfer may be achieved by electrofusion or micromanipulation, the latter being the simplest approach. The micromanipulation technique requires aspiration of cytoplasm containing various nucleic acids, proteins, mitochondria and other cellular organelles from donor oocytes (usually from younger women) and

its deposition into the oocytes of the patient during ICSI. An alternative approach is transfer of the patient's germinal vesicle (GV) to an enucleated immature donor oocyte [15]. Amid concerns over the safety of cytoplasmic transfer [16], and following the birth of three children exhibiting developmental disorders (Turner's syndrome in two and an autism spectrum disorder in the other), the US Food and Drug Administration (FDA) effectively banned such treatment in 2001 until such time that its safety could be clinically proven.

Similar to cytoplasmic transfer, mitochondrial replacement therapy (MRT) has been recently introduced to alleviate the maternal inheritance of mitochondrial disease [17]. The objective of MRT is to transfer genetic material from a patient's oocyte into an enucleated donor oocyte, devoid of mitochondrial genetic mutations. There are various ways in which this might be achieved including polar body transfer (PBT), maternal spindle transfer (MST) and pronuclear transfer (PNT), MST and PNT having recently been approved for MRT by the Human Fertilisation and Embryology Authority in the UK.

36.4 Troubleshooting

36.4.1 Micromanipulators

If it proves impossible to bring the injection micropipette into focus at the surface of the microinjection dish, the most likely reason for this is that the angle of alignment of the micropipette is too obtuse, causing it to be 'heel-down' and pushing the pipette tip upwards and out of the microscope's focal range at high magnification. NB. Attempting to raise the focal plane will bend the micropipette further until it eventually snaps. The remedy for this problem is to lower the focal plane until the micropipette is clear of the surface of the microinjection dish and then use the angle compensation screw of the PL30 tool holder (Fig. 36.2) to adjust the pitch angle to a steeper position.

If the micropipette fails to move smoothly in either the X or Y plane, the most likely reason for this is that its tip is scraping along the surface of the microinjection dish, resulting in a juddering movement. This is simply remedied by raising the micropipette off the surface of the microinjection dish using the fine control lever of the micromanipulator (Fig. 36.3).

If there is no movement in the Z plane in response to rotation of the fine control lever of the micromanipulator, the most likely reason for this is that the control lever has reached the limit of its travel. This is simply remedied by resetting the fine control lever to its mid-point by rotating it in the opposite direction in order to free the locked movement.

If the fine or coarse control levers are too stiff or loose, the most likely reason for this is that the ball joint is out of adjustment. The remedy for this is to loosen or tighten the screws in the plate that retains the ball joint in place (Fig. 36.8) using the appropriately sized hexagonal wrench supplied with the Integra3™.

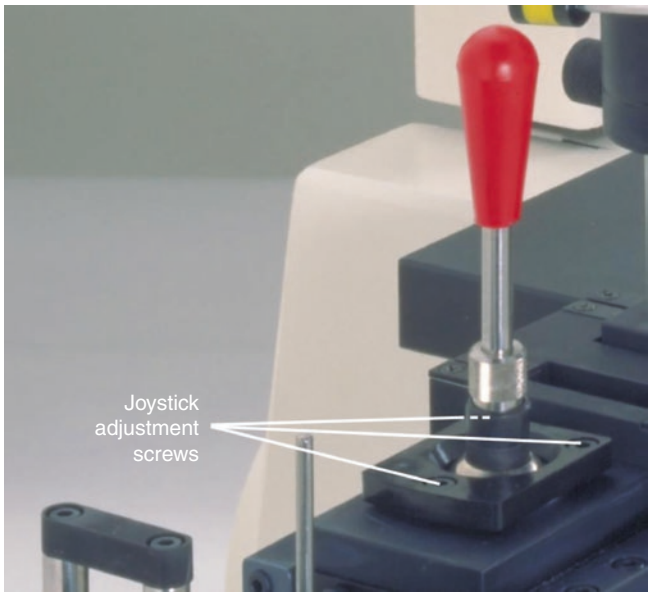

Fig. 36.8 Joystick ball joint retaining plate adjustment screws

36.4.2 Microinjectors

Should it prove impossible to control the sperm's position within the injection micropipette, the most likely cause of this is incorrect priming and equilibration of the micropipette. In this event, it will be necessary to repeat the steps described in the 'Equipment installation and set-up' section above.

Should there be drifting of the sperm's position following correct priming and equilibration of the injection micropipette, then the most likely cause of this is an air leak. In this case, check the tightness of both the MPH microtool holder and microinjector seals. If necessary, cut a 10 mm length off the end of the polythene tubing and reconnect it to create a fresh seal. If this fails to resolve the problem, remove the top of the SAS microinjector and replace the O-ring inside the barrel, and lubricate the O-ring using the special lubricant supplied by RI.

Review Questions

1. Can you list at least three micromanipulation techniques that are possible to be performed using RI micromanipulation systems?

2. What is the likely cause for a micropipette failing to move smoothly in either the X or Y plane of movement?

3. What are the indications that the sperm plasmalemma has been ruptured prior to microinjection, and why is rupture of the sperm plasmalemma biologically relevant?

4. Which checks should be performed in the event that there is drifting of the sperm's position following correct priming and equilibration of the injection micropipette?

5. Can you list at least three reasons why trophectoderm biopsy of blastocysts is preferable to blastomere biopsy of cleavage-stage embryos?

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Eppendorf Micromanipulator: Setup and Operation of Electronic Micromanipulators

Laszlo Nanassy

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Learning Objectives

- To describe the Eppendorf manipulator system and its history
- To highlight the main differences between the Eppendorf manipulator and other systems
- To provide detailed information about assembling and setting up the Eppendorf system
- To provide useful tips on how to operate the Eppendorf micromanipulation system efficiently

37.1 Eppendorf as a Company

The roots of today's Eppendorf AG can be traced back to Dr. Heinrich Netheler and Dr. Hans Hinz, who formed "Dr. Netheler's work group" in 1945 in a building on what was then the campus of University Medical Center Hamburg-Eppendorf. The workshop was established under the supervision of both scientists in order to return the hospital to a functioning state after the war. The team succeeded in repairing many devices and invented new ones like Thermorapid and Eppendorf photometer. Since then, Eppendorf AG, Hamburg, Germany, has created many devices and tools which are now considered breakthroughs in the field of laboratory applications. Today Eppendorf is a leading life science company that develops and sells instruments, consumables, and services for liquid handling, sample handling, and cell handling in laboratories worldwide. Its product range includes pipettes and automated pipetting systems, dispensers, centrifuges, mixers, spectrometers, and DNA amplification equipment as well as ultralow-temperature freezers, fermenters, bioreactors, CO_2 incubators, shakers, and cell manipulation systems. Consumables such as pipette tips, test tubes, microliter plates, and single-use bioreactor vessels complement the range of highest-quality premium products.

Eppendorf products are most broadly used in academic and commercial research laboratories, for example in companies from the pharmaceutical and biotechnological as well as the chemical and food industries. They are also aimed at clinical and environmental analysis laboratories, forensics, and at industrial laboratories performing process analysis, production, and quality assurance. Eppendorf has about 3000 employees worldwide. The company has subsidiaries in 25 countries and is represented in all other markets by distributors.

37.2 The History of the TransferMan

Eppendorf has a long history of micromanipulation equipment that started in 1982 when the company received a letter from the European Molecular Biology Laboratory (EMBL) in Heidelberg, Germany, requesting for a "device for injection of very small (approx. 10^{-15} L) volumes, which (they) employ with great success in the needle microinjection into living cells." After testing the first prototypes in 1984, the microinjector 5242 was launched replacing the barely reproducible and laborious manual work with a glass syringe. With the help of joystick-controlled stepper motors, a very precise positioning was possible when in 1988 the successor model was launched. A revolution was the coordinated process of manipulation and injection which was enabled by a communication protocol of both devices. In 1996, the first TransferMan was introduced. It was already equipped with specialized features for different working fields. An advanced version of the TransferMan was the TransferMan NK (1999) that was equipped with a proportional joystick instead of a dynamic which simplified IVF workflows. Its first successful application of intracytoplasmic sperm injection (ICSI) [1] showed its effectiveness [2] and had been adopted by many scientists. A micromanipulator's main usage in ART was for assisted fertilization, but many other procedures could be performed with it such as assisted hatching [3] or embryo biopsy [4], especially operations requiring proportional movement like ICSI. The newest manipulator generation, the TransferMan 4 model, launched in 2014, combines an intuitive user interface with an unprecedented movement control.

37.3 Description

37.3.1 Components

The Eppendorf TransferMan 4m (Fig. 37.1) is composed of two main components: the motor module unit (Fig. 37.2) and the control board (Fig. 37.3). The control board is mechanically separated from the motor module.

37.3.2 Motor Module Unit

The motor module unit can be fixed on any side of a microscope. For best results, especially for ICSI procedures or any cell surgery, the motor modules are mounted via a microscope-specific adapter. The motor module is made up

Fig. 37.1 The Eppendorf micromanipulator is composed of two main components: motor module unit and control board

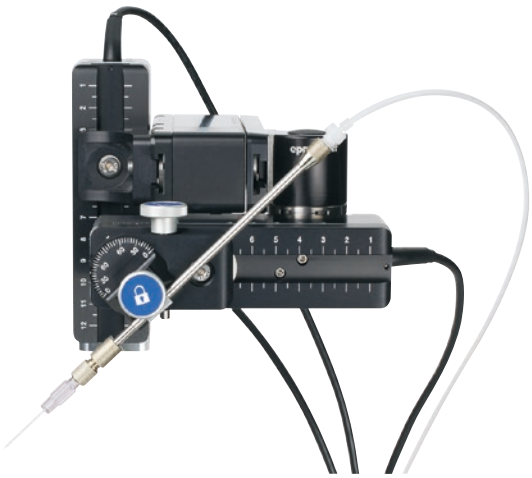

Fig. 37.2 Mounted motor module with attached capillary holder

Fig. 37.3 Control board of Eppendorf TransferMan 4

of three modules for the x-, y- and z-directions. The capillary can therefore be moved in all three spatial axes. The x-module with the capillary can be swung out of the working range using the swivel joint. This allows an easy capillary exchange. The injection angle of the capillary can be set on the angle head.

37.3.3 Control Board

The upper part of the control board contains the joystick which enables the user to make horizontal and vertical movements in a proportional and dynamic way. This unique DualSpeed™ movement gives precise, instantaneous control and positioning using different speed modes. The position can be changed to another saved position by pressing the button twice. The working range can be chosen between coarse and x-fine mode depending on the needed travel distance of the capillary. The speed is adjusted accordingly. One of the main advantages of the Eppendorf micromanipulator is that it is user-friendly even for new users. It can save certain positions and allows for rapid changing between them. In addition, a z-limit can be programmed, limiting pipette movements downward and maintaining the distance to the Petri dish. The position feature is not found in other brands of micromanipulators used in IVF and greatly reduces the risk of pipette breakage by inexperienced and experienced users. The "home" button allows fast withdrawal of the pipettes from the stage, allowing for changing the sample or pipette. Pressing the "home" button again returns the pipettes to the previous position. The display illustrates the axis coordinates and the options chosen. The multifunctional keypad gives the desired function when the button is pressed or released and allows multiple positions to be saved. With the selection dial on the side, the working range can be easily adjusted. In the rear part of the control board, the connections for the motor modules and external devices are located. Also, the power connection is located there.

37.4 System for Micromanipulation

Micromanipulators are one of the main components of a manipulation workstation. Therefore, they have to be carefully chosen to ensure the best possible results. In addition, it is important to choose appropriate optics for embryo manipulation for the inverted microscope. Also, careful consideration is needed when a heating stage is chosen. It has to be reliable and keep temperature within a range that is not damaging the cells and provides a uniform heat distribution. It is also highly recommended to use an anti-vibration table or alternative solution (e.g., anti-vibration pads) in order to avoid unnecessary damage during manipulation.

The location of the workstation within the laboratory is also worth considering. It is recommended to place it close to the biosafety cabinet and incubators and far from air ventilation systems if possible. The ergonomics of the station is also important as long hours of work are expected on the manipulation station.

37.5 Setting Up the Hardware

Before setting up, as always, it is advisable to read the manual of the equipment. Usually, the manufacturer assembles the manipulator in the laboratory and sets up working units, but fine alignment is the responsibility of the user.

The first step with the Eppendorf system is to center the x- and y-motors and to drive the z-motor down by 80%. Then, with capillaries inserted into the injection holders, both modules should be positioned resulting in needles being in the middle of the field of view. In order to make sure that we have the tips of the needles at the same position or nearby at every setup, the depth of the needles can be fixed with the adjustable clamp on the injection holder. It has to be noted that the needles are needed to be pushed into the holders to the endpoint in order to be able to reproduce the setup every time.

Obviously, the angle of the needles needed to be set up based on the choice of needles. The final step is to save the centered set up that can be recalled when needed. Before every manipulation, only fine alignment is necessary; thus, a fast setup can be accomplished.

37.6 Setting Up the Software

As mentioned previously, in the case of the software, it is also worth studying the manual as it can make the operator's life easier. There are different applications preprogrammed based on the purpose of the procedure. We can find an application for ICSI where there are two main functions preset, the Y off and the Z-axis limit options. Also, there is a possibility to add other user-specific applications since additional spaces are available in the software. Other predefined applications such as "cell transfer" and "DNA injection" can be chosen and further fine-tuned based on the operator's preferences. Another option is to choose "My application" which is a fully programmable application.

37.7 Training on TransferMan

Like with all manipulators, operators need some training to be able to work efficiently on the Eppendorf units.

Motors are strong enough not to feel any resistance if we reach the bottom of the dish and needles can be broken. In case of other manipulators, this phenomenon could be more obvious. It needs to be visually checked when dealing with the depth of the capillaries.

The working range can be changed, which is the amount of movement of the needles that is translated by the movement of the joystick. This is solely based on the preference of the operator but needs some practice to work out the most optimal setting. There are three main options (coarse, fine, and extra fine), each of which can be changed using the selection dial on the left side of the control panel. Another feature that is different from other manipulators is that the TransferMan 4 allows for proportional and dynamic movement. When the joystick is pressed against the rim on the side, the pipette moves continuously. This function can be disabled based on the operator's preference. It is useful when needles need to be centered.

Also, joysticks can be centered without moving needles. This is a useful tool when fine alignment of the capillaries is needed in the middle of a procedure. Uncoupling the joystick from the motor module is accomplished by pushing and holding the button on the top.

In order to be able to acquire movement only for one direction, the "Y off" function can be enabled. This function is similar to what the Narishige system is capable of when only rotating one knob, for example for injecting the spermatozoon into the egg. This function is preset when the ICSI application is chosen on the Eppendorf system.

A vertical limit of the capillary movement can be set. For this option, when the desired lowest point is reached, the "Z-axis limit" function should be enabled. Although this is a useful option, it should be noted that dishes used for ICSI are not always plain enough to be able to take advantage of the function.

One simple feature that can fasten procedures on Eppendorf manipulators is the double click on the joystick button. This allows the user to switch between saved positions (e.g., inside and above the injection droplet of the ICSI dish).

37.8 Everyday Work

It is the responsibility of every operator of the ICSI station to maintain the proper functionality of the equipment. For efficient manipulation work, it is crucial to properly set up our system. When the instrument is turned on, the centered setup can be recalled if it was saved as one of the positions. If not, all motors can be centered from the main menu (menu/function/executing center motors). It is advisable to set the microscope to the lowest magnification. Before mounting pipettes, both manipulator arms can be lifted by pressing the "home" buttons. The X-module can be swiveled out, allowing for an easier access to inserting the capillary. After inserting capillaries on both sides, the manipulator arms can be set to the previous position by pressing the "home" buttons again. If the adjustable clamp on the injection holder is set to the right depth, the injection holder can be slid down gently to its endpoint. We should have a more or less centered position of capillaries in the plain, and only fine adjustment should be necessary. Then the alignment should be checked at the magnification at which the actual work will be carried out. After finalizing the alignment of the pipettes, this position can be also saved by pressing and holding the "Pos" button. This should be saved in a different position than zero or centered setup. With this, we can recall this setup any time during the manipulation procedure if it becomes necessary. Between patients, the centered setup can be recalled, and after fine alignment, the setup can be saved again. Both manipulator arms can be lifted by simply pushing the "home" button, and the dish can be placed on the microscope stage to start the procedure.

37.8.1 Priming of Capillaries

Eppendorf offers two types of injectors (air and oil) that can be used on both the holding and the injection side. Injectors from all other manufacturers are also compatible with the Eppendorf system. Thus, operators with certain injector preferences can use this system as well.

Oil-based injectors are able to offer a very fine control. In these injectors, the tubes are filled with oil that cannot be compressed, which translates the movements to the tip of the needles. Before capillaries are inserted, oil should be pushed through the pipette holder to make sure that there is no air in

the system. The transparent cylinder of oil-based injectors should be visually checked prior to work every day if it is free of any air bubbles. It is also critical to ensure there is enough oil in the system and to refill if necessary. For this, the filling screw should be unscrewed from the filling bore and a syringe filled with oil attached and cylinder filled with oil while the injector is held vertically.

Priming of capillaries is simple; the medium can be withdrawn to a point that still can be observed under the microscope at a lower magnification. Suction should be stopped by turning the knob in the opposite direction, and basically priming of the capillaries is done.

A similarly fine control of samples can be obtained using air-based injectors as well, but priming of capillaries needs to be done differently. Usually, for the holding side, the capillary action is enough to prime. The capillary needs to be submerged into the medium and equilibrated for a couple of minutes. The level of the medium should be in the vicinity of the middle of the pipette. For embryo biopsies, this should be sufficient to prime the injection side as well depending on the diameter of the biopsy pipette. If it is narrow, a little suction can be applied, and waiting a little is recommended because of slower equilibration. For ICSI, first priming is needed with the medium applying suction and waiting a couple of minutes. Then, priming is needed with PVP or any other medium that is used for slowing down sperm.

Less attention is needed when working with air-based injectors. It is worth checking the injectors before manipulation if there is enough room in the cylinder for priming and performing the actual procedure.

37.8.2 ICSI, Embryo Biopsy, and Other Techniques

The detailed description of executing ICSI, embryo biopsy, or other manipulation techniques is beyond the scope of this chapter.

Probably, the most common manipulation procedure is still the intracytoplasmic sperm injection. For ICSI, on the injection side, it can be helpful to set the angle a bit steeper to obtain easier sperm handling. The angle can be easily changed with the knob on the top of the angle head.

37.9 Maintenance

Eppendorf does not specify certain time frames for service. Service is needed only if problems arise. Similar to every precision instrument, service needs to be done by professional Eppendorf technicians.

Review Questions

1. How is the Eppendorf manipulator system different compared with other systems?
2. What are the specific functions of the Eppendorf system that are most helpful for operators during the manipulation of oocytes and embryos?

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Oocyte Treatment and Preparation for Microinjection

Thomas Ebner

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Learning Objectives

- To learn how to score cumulus-oocyte complexes
- To learn how to avoid lethal stress during the denudation process
- To realize the potential of cumulus cells as predictors of oocyte quality
- To keep in mind the physiological way of sperm processing and selection
- To learn how to perform ICSI in the presence of immotile sperm
- To learn how to deal with cases of ICSI fertilization failure
- To learn when a modified ICSI technique is useful

It must be emphasized in advance that two major problems must be solved in preparation for ICSI. First of all, it has to be ensured that temperature is constantly kept at physiological ranges (approx. 35–37.5 °C) throughout the whole process of oocyte collection, denudation, and injection. For this purpose, the temperature of all laboratory devices involved (e.g., heating plate, transportable container, microscope stage, incubator), has to be set higher than the requested temperature in the prevailing incubation medium. The required difference has to be tested individually for each laboratory set up and will be closely related to the volumes of culture medium and/or mineral oil used.

The second prerequisite for an optimal ICSI performance is a well-calculated time schedule. The period between oocyte collection and subsequent ICSI should never exceed 6 hours [1, 2] in order to avoid in vitro aging of the oocytes. In this respect, it seems irrelevant whether the cumulus-oocyte complexes (COC) are denuded immediately after collection leaving denuded eggs in culture for later ICSI or if the manipulation is performed directly prior to ICSI after a resting period of several hours [1]. However, since oocytes may develop osmotic problems, e.g., an unwanted influx of culture medium, after a prolonged period without cumulus cells attached (unpublished data), and cumulus cells play an important role in maturation of the oocyte, it is recommended to perform processing of the COCs close to the time of injection.

38.1 Processing of the Cumulus-Oocyte Complex

Harvested COCs are traditionally evaluated by their appearance and by the expansion of the corona radiata and the cumulus complex. Based on these criteria oocytes within cumulus matrix are roughly categorized as either mature (metaphase II) or immature (pro- and metaphase I). In detail, an expanded and luteinized cumulus complex and a radiant corona radiata suggest completion of nuclear maturation, while the absence of expanded cumulus or corona cells is associated with immaturity [3]. This is in contrast to more recent data which show that nuclear maturation and oocyte quality cannot be predicted adequately by scoring the COCs [4, 5].

Frequently, blood clots or other amorphous clumps [5, 6] are present in the cumulus matrix. Embryologists tend to cut off these areas with the help of two needles. Apart from the fact that this is mechanical stress to the oocyte, particularly if the dysmorphic cells are close to the corona radiata, it is of no use. On the one hand, amorphous clumps, considered to be a sign of postmaturity, did not have any effect on preimplantation outcome, and on the other, COCs showing blood clots have already been harmed during folliculogenesis and, thus, their developmental capacity may not be retained by cutting off the blood clots mechanically [5].

38.2 Denudation of the Oocyte

For successful ICSI it is critical that cumulus cells are adequately removed from the oocyte. Apart from the fact that oocyte maturity and/or quality need to be checked prior to injection, some technical problems could occur. Theoretically, it could happen that the oocyte cannot be manipulated adequately with the holding pipette if these nutritive cells block the 9 o'clock position. In addition, there is a certain risk to accidentally bring in foreign somatic DNA into the egg if cumulus cells are still attached at the site of injection [7].

It has been established that any denudation process consists of two steps, an initial enzymatic digestion followed by mechanical precision work. Since it has been shown that a dislocation between the first polar body and the meiotic spindle could occur if the mechanical part is performed inadequately, e.g., using pipettes of an inappropriate inner diameter (<140 μm), it is recommended to prolong the enzymatic incubation period. This would definitely help to minimize the mechanical denudation part, in particular to reduce shear stress [8].

Usually, commercially available hyaluronidase is used to start the denudation process. Hyaluronidase is an enzyme degrading hyaluronic acid which is a major component of the extracellular matrix of the oocyte. Most of the commercially available hyaluronidases have a concentration of 80 IU/l, which is only a tenth of the critical threshold above which parthenogenetic activation might occur [9]. For reducing the theoretical risk of harming the oocyte even further, incubation time could be shortened (30 seconds) or the dilution could be changed to 40 IU/l (1:1 mixture with culture medium). Ultimately, it seems to be sufficient to put few drops of hyaluronidase in the well containing the COCs, but in such a case, the exposure time will naturally need to be prolonged to approximately 10–15 minutes (P. Vanderzwalmen, personal communication).

Alternatively, plant (CoronaseTM, Bio-Media, Boussens, France) or recombinant human products (ICSI Cumulase™, Origio, Måløv, Denmark) could be applied [10]. It has been argued that due to the reduced toxicity of these enzymes, exposure time is not critical anymore.

The question remains if it is at all necessary to completely denude the female gametes. Our study group could demonstrate that coculture using homologous cumulus cells in situ

(partial denudation) is associated with an enhanced rate of in vitro maturation, embryo quality, and blastocyst formation [11]. Obviously, leaving numerous cumulus cells attached to the zona pellucida mimics the in vivo situation and utilizes the stimulatory effect of the somatic cells during the first days of preimplantation development. However, due to their varying stage of maturity oocytes do not present a standardized pattern of cumulus cell attachment. Younger oocytes (from the time of ovulation induction) show a more homogeneous pattern with cumulus cells involving the whole surface of the gamete, which makes ICSI somewhat difficult since there is almost no cumulus cell-free access to the zona. Indeed, it has been suggested that incomplete denudation of oocytes might increase degeneration rate under certain circumstances [12].

38.3 Cumulus Cells as Noninvasive Predictors of Oocyte Quality

It should be emphasized in this context that the detached somatic cells could be used for both adequate prognosis of the quality of the associated oocyte and treatment outcome. All techniques available, e.g., analysis of apoptotic phenomena [13], evaluation of telomere length [14], or cumulus cell gene expression [15], are based on the hypothesis that a healthy follicle gives birth to good-quality gametes and granulosa cells. Female gametes are further thought to protect the associated cumulus cells since inside-out gradients in gene expression of bone morphogenetic proteins 6, 7, and 15 [16] and DNA-integrity [17] have been observed.

38.4 Catching of the Spermatozoa

In parallel to the processing of the COCs, the ejaculate produced under sterile conditions has to be processed for further usage. This can either be done using a centrifuge combined with a swim-up procedure (e.g., Percoll, density gradient, Sephadex columns, glass wool column) or sperm isolation without centrifugal stress (Zech-Selector, microfluidics).

Since in most of the patients a certain proportion of sperm is motile, embryologists are faced with the problem of catching these gametes prior to immobilization and injection. This procedure can be performed in three different milieus: culture medium, polyvinylpyrrolidone (PVP), or a more physiological viscous solution.

Naturally, catching sperm in the same balanced culture medium in which the oocytes are cultured (different drop) would be the most natural approach. Loading a processed sperm sample into medium drops on an ICSI dish has the benefit that motile sperms will automatically separate from immotile gametes, somatic cells, and other debris by their motility. This makes the purity of the whole drop much higher and minimizes theoretical contamination. However, this mode of catching sperms has the drawback that without a viscous solution covering the inner surface of the injection pipette sperm manipulation during injection is not a smooth process but much rather a jerky one. This problem cannot be overcome, but it can be reduced by either washing the ICSI tool with PVP or by slightly changing the injection technique [18]. It is recommended that a minimum volume of medium is placed in the ICSI pipette (border between medium and oil/air should be below the knee of the pipette) and that the sperm is placed in the foremost third of the visible part of the glass tool. These precautions will facilitate stabilization of the sperm and help further manipulation. Embryologists using this setup often tend to aspirate the sperm with its head first since this is much easier and less time-consuming than trying to aspirate a hypermotile sperm from its tail end. Although ICSI in reverse has been found to be of similar outcome [19], there is a much higher risk of artificially creating vacuoles at zygote stage [20] since the larger volume (as compared to sperm head-first ICSI) of medium entering the oocyte frequently becomes encapsulated (Fig. 38.1).

For these reasons, most embryologists regularly use a viscous water-soluble polymer (PVP) of the monomer N-vinyl pyrrolidone to facilitate manipulation of sperms (and to coat the injection pipette). In contrast to the usage of culture medium, PVP that enters the oocyte cannot be actively removed through membrane channels due to its rather high molecular weight (between 40,000 and 360,000). Thus, larger volumes of PVP could alter intracytoplasmic pressure and/or osmotic behavior of the oocyte. It has to be mentioned that PVP per se is not toxic at all, e.g., it is used in personal care products such as toothpaste, contact lens solutions, and shampoo. However, there is no denying the fact that it is an unphysiological liquid.

This circumstance led several companies to introduce more physiological solutions for sperm manipulations. Currently, two such products are on the market, SpermSlow™ (Origio, Måløv, Denmark) and SpermCatch™ (Nidacon, Mölndal, Sweden). Both are based on the finding that a

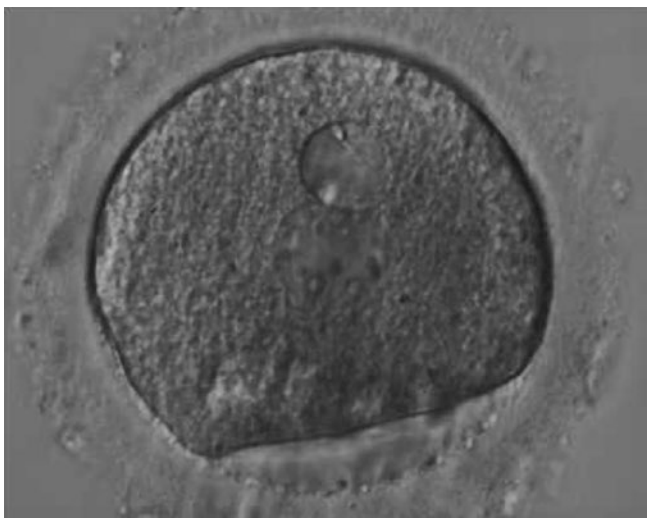

Fig. 38.1 Artificially created vacuole (V) after ICSI (day 1) resulting in a monopronuclear (Pn) zygote. Please note that sperm is located within the vacuole

naturally occurring major component of the cumulus cell matrix called hyaluronate (polymeric chain of glycoseaminoglycans) slows down the movement of spermatozoa and could act as a natural alternative to PVP [21]. Hyaluronate has a relatively high negative charge and a high hydration capacity that allows for the preparation of solutions with adequate viscosity for ICSI. It has been reported that its effect on sperm motility is reversible, and its use does not affect the outcome of the treatment cycles in terms of fertilization, pregnancy, and live birth rates [22]. However, it has to be noted that hyaluronate-based products do not have the same effect on the ease of sperm modulation as PVP probably due to its rather low content of hyaluronate (approx. 1%).

38.5 Immobilization of the Spermatozoon

Once the spermatozoon is caught, it appears necessary to immobilize it prior to injection [23]. Regardless of the fact that under normal in vivo conditions no sperm tail enters the oocyte, immobilization of the sperm has two beneficial effects: on the one hand, any possible damage to the cytoskeleton caused by motile sperms is theoretically negligible, and on the other, permeabilization of the sperm membrane will ensure that a soluble oocyte-activating factor (phospholipase C zeta) immediately enters the ooplasm [24].

Sperm immobilization is usually performed toward the end of the tail (back half); however, permeabilizing alternative sites is also possible. Yong et al. [25] successfully damaged the head membrane of porcine spermatozoa. This is also possible in the human since sperm chromatin is tightly complexed to protamines (approx. 85%) and histones (approx. 15%) and further stabilized by the formation of intra-intermolecular disulfide cross-links between the cysteine residues of the protamine molecules [26]. Thus, any suggested mechanical harm to sperm DNA is only of theoretical nature, e.g., laser shots directed at the sperm head did not cause DNS strand breaks (unpublished observations).

However, sperm immobilization can be performed using four different methods. The most common approach would be a mechanical one, e.g., pressing the tail of the spermatozoon to the bottom of the ICSI dish by use of the injection pipette [23]. This is in line with the work of Palermo et al. [27] who found a more aggressive mechanical immobilization process in epididymal sperms helpful in order to increase fertilization rate from 48% to 82%.

Sometimes the angle of the ICSI pipette is suboptimal, permitting no adequate manipulation of the spermatozoon. In such cases, repeated aspiration in and out the injection pipette is found to be helpful to immobilize sperms. However, only 16% of the corresponding oocytes showed 2Pn as compared to conventional mechanical breakage (90%) of the sperm membrane [23].

Montag et al. [28] introduced laser-assisted permeabilization of the sperm membrane into the field of assisted reproduction. Our study group [29, 30] successfully used this mode of immobilization as a routine procedure. In detail, spermatozoa were immobilized with a noncontact diode laser (1.48 µm wavelength) applying a double shot strategy. Two successive laser irradiations were applied per spermatozoon, the first aimed near the middle of the tail (1.5 mJ) and the second directly at the end of the tail (1.0 mJ). This strategy minimized the total energy dose male gametes were exposed to. In addition, laser shots were placed far from the head, which made laser application for immobilization a presumably safe process.

A fourth alternative is piezoelectric manipulation of the motile sperm [31]. The same authors [32] published that the piezo method shows the most rapid onset of Ca^{2+} oscillations of all techniques (except laser immobilization) and, thus, may have caused the most damage to the sperm membrane. The method of sperm immobilization may be important for the rapid release of sperm factors that initiate oocyte activation.

38.6 Selection of Spermatozoa

Whatever method appears convenient, special care should be taken to select spermatozoa with best prognosis in terms of fertilization and further preimplantation development. Optimal selection of male gametes is a prerequisite for a successful ICSI program and should at least be performed at a magnification of ×400 if not at much higher magnification [33]. Not only should embryologists accurately evaluate normal sperm morphology [34, 35] in order to use gametes of optimal prognosis, but it is also of utmost importance that these cells reveal a high grade of maturity and genetic stability.

Huszar et al. [36] reported that a hyaluronate receptor is expressed in mature spermatozoa only after plasma membrane remodelling during spermiogenesis, and that hyaluronate is an ideal medium for sperm selection for ICSI. Thus, hyaluronic acid (HA) has recently been used as "physiologic selector" for spermatozoa prior to intracytoplasmic sperm injection as a convergence to a more physiological fertilization [22, 37, 38]. Spermatozoa bound to HA show a significant reduction in DNA fragmentation and a significant improvement in nucleus normalcy compared with spermatozoa immersed in PVP. Furthermore, injection of HA-bound spermatozoa significantly improved embryo quality and development [22], whereas zygote score was unaffected [37].

It should be noted that HA-ICSI requires special preparation of the ICSI dish. In detail, a small (e.g., 2 µl) droplet with suspension of spermatozoa has to be connected with a pipette tip to a slightly larger (e.g., 5 µl) droplet of HA-containing medium and allowed to incubate for 15 minutes at 37 °C under oil. Thereafter, spermatozoa bound to HA in the junction zone of the two droplets can be detected, easily detached by the injection pipette, and subsequently used for injection.

A similar mode of selection aiming toward more mature spermatozoa utilizes spermatozoa previously bound to the zona pellucida of an immature egg [39]. This specific binding induces the acrosome reaction and, theoretically, should

provide for better ICSI outcome. Indeed, embryo quality was found to be increased, although fertilization rate was unaffected [39].

In practice, it has been suggested [39] that a processed sperm sample (ca. 1×10^6 motile spermatozoa per ml) should be incubated with one MI-oocyte in buffered culture medium. After a 2-h incubation period, the eggs should be carefully washed to dislodge sperms loosely adhering to the surface of the zona pellucida. Spermatozoa bound to the MI-oocyte zona pellucida are presumed to be mature and can be removed with a microinjection needle for subsequent ICSI. However, since MI-gametes did not finish either nuclear or cytoplasmic maturation, it is questionable whether immature eggs express a zona pellucida selecting for the same male gametes as a MII-oocyte. Another technical limitation is the rather strong binding between sperm head and zona. Anyone having tried to detach bound sperm from the outer shell of an ovum will have realized that this requires rather strong suction forces by the ICSI pipette. Moreover, the sperm tail is still intensely motile since this step has to be performed in culture medium and not in viscous solutions.

Although both methods, using zona- or HA-bound spermatozoa for ICSI, will increase the percentage of genetically intact spermatozoa, there is currently no way to completely remove DNA strand break-free from a given processed sperm sample. Recently, our study group [40, 41] evaluated the efficiency of a particular sperm selection chamber (ZechSelector™, AssTIC Medizintechnik GmbH, Leutasch, Austria) with respect to its selection properties in terms of DNA damage. Interestingly, it turned out that these glass or polyethylene chambers exclusively accumulate strand break-free spermatozoa, which for the first time ensures elective usage of DNA-intact sperms for ICSI. Since the ZechSelector™ strictly separates spermatozoa according to their motility/velocity without exposure to centrifugation stress [42], these parameters should be of utmost importance during sperm selection. Obviously, once DNA damage has occurred, both nuclear and mitochondrial DNA will be affected. Any impact on the latter could reduce ATP production and as a consequence sperm motility.

38.7 Immotile Sperm

This selection criterion can of course not be applied if all spermatozoa of an ejaculate are immotile (e.g., Kartagener syndrome, cryopreserved sperm, TESE-material). In this particular case, embryologists have to be aware that it is of serious consequence if they cannot distinguish between immotile and viable sperms, although immotility does not preclude viability. Theoretically, one has four options to solve this tricky problem.

Commonly, the most reasonable approach would be to use the ICSI pipette in order to test the elasticity of the sperm tail [43]. A spermatozoon showing an elastic tail (once being manipulated with a glass tool) is presumed to be more viable than those with more rigid ones. Typically, these nonviable sperms show incapacity to resume the initial tail position once touched by the pipette from the side, and they show a characteristic "rolling" motion when touched from above. However, in the final analysis there is no guarantee that more elastic sperms are viable, e.g., being an osmotically intact cell.

For confirming osmotic capacity sperms can be incubated in a hypoosmotic swelling solution, e.g., a 150 mOsm NaCl solution [44, 45]. Gametes with a functional membrane will undergo swelling of the cytoplasmic space and the sperm tail fibers will curl, whereas those gametes with damaged or osmotically inactive membranes do not show these phenomena. It is important to consider that swollen and curled sperms have to be moved to an isoosmotic culture medium prior to injection in order to facilitate original state and osmotic status.

Recently, a third alternative was introduced [46] suggesting usage of a diode laser in order to assess viability in cases of complete asthenozoospermia. Applying a single laser pulse (1.2 ms) at the very end of the sperm tail (direct method) caused a characteristic curling of the tail end. Since nonviable sperm did not show this phenomenon, this new technique helped to identify spermatozoa with functional integrity of its membrane.

The fourth strategy is the only one allowing for partial restoration of original motility [47]. Pentoxifylline and other caffeine derivates such as theophylline (Spermmobil™, Gynemed, Lensahn, Germany) are inhibitors of phosphodiesterase activity, which enhance motility in spermatozoa. Using this ready-to-use compound Ebner et al. could successfully treat patients suffering from crypto- and azoospermia [48] as well as in a case of retrograde ejaculation and absolute asthenozoospermia [49].

These agents show maximum activity after 10 minutes and an activity phase of less than 2 hours [50]. Because of this immediate and short-term effect, direct addition into the droplet containing the immotile sperms is recommended.

38.8 Intracytoplasmic Sperm Injection

Regardless of whether a motile or immotile sperm is available, ICSI should be performed according to a standardized procedure. To perform ICSI, the oocyte is held in place with a holding pipette at 9 o'clock. The first polar body usually is located on the 6 or 12 o'clock position. As soon as the equatorial plane of the oocyte is focused, the ICSI pipette has to be pressed against the zona pellucida creating a characteristic funnel at 3 o'clock. After penetrating both the zona and the oolemma, a small volume of cytoplasm should be aspirated into the glass tool to activate the egg and to ensure entering of the ooplasm [51]. The single immotile spermatozoa should then be gently placed near the horizontal axis. Withdrawal has to be done carefully to prevent the oocyte from leakage.

Placing the first polar body farthest from the path of the injection needle was thought to protect the meiotic spindle, which is considered to be located in the periphery of the egg subjacent to the first polar body, against mechanical damage

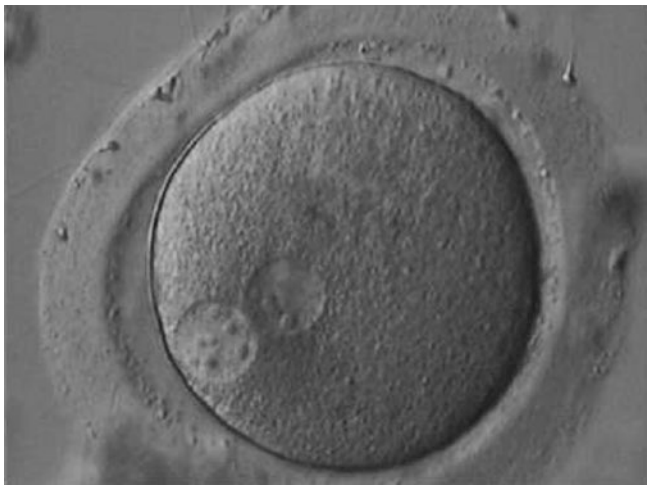

Fig. 38.2 Suboptimal pronuclear formation close to the periphery of the oocyte

[52]. Meanwhile, it has been published that due to the manipulation during denudation, the first polar body is a rather inaccurate marker of the spindle position [53] and that it is almost impossible to harm the dense microtubule structure of the spindle apparatus.

This finding is further supported by data of Blake et al. [54] who analyzed fertilization and embryo development resulting from varying distances between the injected sperm and the polar body associated with the presumed area of the spindle. Among the orientations examined in this paper, depositions of the sperm in the vicinity of a polar body at 9 o'clock resulted in significantly fewer normally fertilized oocytes and significantly more unfertilized and digynic oocytes. All other locations gave similar rates of fertilization and embryo qualities. It appears to be crucial that the immobilized spermatozoon is placed in the very center of the female gamete. Since decondensation of the sperm head and formation of the male pronucleus takes place at the site of sperm deposition [55], any deviation from this optimal place, e.g., close to the periphery of the egg, would result in suboptimal pronuclear formation (Fig. 38.2). This scenario is most likely associated with cleavage anomalies or developmental arrest [56].

38.9 ICSI Failure

Since intracytoplasmic sperm injection is more invasive than other micromanipulation techniques, there is a higher risk of irreversibly damaging the injected oocyte (lysis, shrinkage, and/or tanning of the egg). The rate of degenerated oocytes after ICSI should be around 1% and not exceed 3%. Though most embryologists have made the experience that a suboptimal injection technique may influence ICSI outcome as they progressed on their learning curve with micromanipulation, only few studies deal with degeneration of oocytes [57]. The main problems found were as follows: (i) spermatozoon remained attached to the ICSI pipette while being released, (ii) insufficient immobilization of the spermatozoon (as assessed by subsequent movement of the tail after injection), (iii) rejection of spermatozoon into perivitelline space after ICSI (as assessed by the sperm's tail protruding out of the oolemma/zona pellucida), and (iv) difficult breakage of oolemma. None of the abovementioned deviations from a presumed optimal injection procedure are significantly correlated with oocyte survival except the latter one.

During ICSI, different responses of the zona pellucida and the membrane to the injection pipette can be observed. In contrast to the very frequent normal response, showing a distinct injection funnel prior to rupture, two rather rare breakage patterns are considered as abnormal [27], namely, sudden breakage without any invagination during injection and difficult breakage characterized by delayed rupture of the oolemma. Recently, it could be shown that the characterization of an injection funnel during ICSI reflects cytoplasmic maturity of the oocyte [58], in a way that the more immature an oocyte is, the smaller is the injection funnel, and consequently the ooplasmic viscosity.

It has been shown that additional manipulation in MII oocytes showing difficult oolemma breakage may cause an increase in degeneration rate [59]. In order to avoid this scenario, a modified injection technique has been suggested [52] combining a pressing and a sucking phase, thus keeping oocyte survival rate at an adequate level.

38.10 Laser-Assisted ICSI

To overcome this high risk for degeneration in such oocytes, an alternative laser-assisted intracytoplasmic sperm injection has recently been suggested [60] and successfully applied in patients with diminished oocyte survival in previous cycles [60, 61]. This method involves injection of the oocyte through a small laser-created hole (5–10 μm) in the zona, which facilitates penetration of all anatomical structures. As a consequence, oocyte survival is increased significantly, as demonstrated in a larger number of cases [62].

However, none of the abovementioned studies took into account a major problem of laser-assisted ICSI, namely, the impossibility to localize the laser-generated hole at later developmental stages [62]. This phenomenon is particularly evident at the blastocyst stage, when the embryo expands and the zona pellucida gets thinner prior to hatching. Thus, if assisted hatching is applied in such embryos, as recommended in embryos derived from oocytes with difficult penetration of the oolemma [63], an additional opening is unintentionally created, which might impair the hatching process per se and/or result in monozygotic twinning [64].

In order to avoid this possible dilemma, Moser et al. [65] decided not to perform ICSI through a relatively small opening but through a zona pellucida area on which laser zona thinning [66] was applied. This approach allows for accurate location of the manipulated zona area at later developmental stages and, theoretically, should combine two advantages, namely, minimal mechanical stress to the oocyte during ICSI (e.g., increased oocyte survival) and assisted hatching.

Laser-assisted ICSI suggests that difficult penetration during ICSI is always caused by the zona pellucida and never by the structure of the oolemma. As in zona-free ICSI no injection funnel forms in laser-assisted approaches and immediate penetration is observed.

38.11 Modified ICSI

Considering the complexity of the fertilization process may help to understand its susceptibility to disturbances potentially causing complete fertilization failure (in spite of the presence of a presumably normal spermatozoon). The frequency of total fertilization failure cycles is up to 3% with most of them being the result of impaired semen characteristics or a very low number of eggs collected. In such cases, repeated ICSI treatment proved useful [67]; however, some patients will have to face repeated fertilization failure in spite of normal sperm parameters and good ovarian response.

In order to rescue such cycles, Tesarik and coworkers [68] reported a modified intracytoplasmic sperm injection technique mainly based on a repeated dislocation of central ooplasm to the periphery, thus increasing the intracellular concentration of free calcium by either creating an influx of calcium ions or a considerable release of calcium stored in cell organelles.

Taking into account a possible negative effect of this rather vigorous injection technique on further preimplantation development, another modified ICSI version was developed [69], which is based on the hypothetical accumulation of high-polarized mitochondria, e.g., showing a high inner mitochondrial membrane potential [70], from pericortical regions (9 o'clock) to the center of the oocyte, thus supplying more energy (ATP) directly to the place where the spermatozoon is normally injected. In this respect, it proved helpful that aggregation patterns of mitochondria correspond well to the light-microscopical appearance of the oocyte [71]. In 17 cases of complete fertilization failure after ICSI, we [69] could achieve a 54% fertilization rate and a 33% clinical pregnancy rate, respectively. However, it must be emphasized that the positive effect of our modified ICSI that could be shown in cases of previous fertilization failure after standard ICSI could not be demonstrated in cases without this problem since fertilization rate and further development were comparable. This implies that a minimum baseline of functionally active mitochondria must have been present in oocytes without impaired fertilizability [72].

More recently, an additional modified ICSI technique has been suggested [73], namely, increasing the effectiveness of ICSI by piezoelectric activation. In 50 patients with more than one previous total fertilization failure after ICSI, as many as 48% eggs could be fertilized resulting in a 44% clinical pregnancy rate.

Since all these techniques to overcome fertilization failure after ICSI are either rather invasive or require certain technical skills or equipment, usage of a calcium ionophore, e.g., Calcimycin (CULTactive™, Gynemed, Lensahn, Germany), can be recommended in such patients [74]. This ready-to-use solution (Ca^{2+}-ionophore in DMSO and culture medium) opens membrane channels and facilitates entrance of extracellular Ca^{2+} from the culture medium, which is a prerequisite for oocyte activation and fertilization. In fact, it proved useful in cases of complete fertilization failure or reduced fertilization after ICSI [75], severe male factor infertility [76], and developmental problems/arrest/delay [77].

In practice, immediately after ICSI (since the ionophore might alter the constitution of the zona), injected oocytes are incubated in a bath of ionophore (approx. 15 minutes), whereafter the ionophore has to be removed by carefully washing the ova several times.

38.12 Conclusion

ICSI is undoubtedly one of the most severe manipulation techniques in IVF laboratories. Not only does it require a certain learning curve, but it is also particularly dependent on oocyte quality, which is governed by individual patient response and other stimulation details. It is a fact that suboptimal ICSI results in an impaired oocyte survival and reduced preimplantation development [57, 78]. It is a prerequisite to use ICSI pipettes of standardized quality that show a relative sharp spike (Gynemed, Lensahn, Germany; Humagen, Charlottesville, VA, USA). These tools combined with the individual skills of the embryologist will maximize fertilization rate and outcome, particularly if only a limited number of female gametes is available. In difficult ICSIs, spontaneous change of the injection technique can rescue the cycle or optimize the results. Additional help may come from the usage of theofylline, Ca^{2+}-ionophore, and (in the case of TESE) collagenase (Gynemed, Lensahn, Germany). To conclude, there is a general tendency toward the application of a more physiological ICSI using PVP substitutes (Origio, Måløv, Denmark; Nidacon, Mölndal, Sweden) and more mature and strand break-free spermatozoa.

Review Questions

1. What are the potential pitfalls in oocyte denudation?
2. Which techniques can be applied to cumulus cells in order to predict the health of the associated oocyte?
3. Which different milieus would facilitate sperm processing?
4. Which sperm immobilization techniques are reported in literature?
5. How can spermatozoa be selected physiologically?
6. How can immotile sperm be treated in order to restore motility?

7. What should be avoided in ICSI?
8. How can ICSI failures be overcome?
9. In which situations are modified ICSI techniques recommended?

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Piezo-ICSI

Kenichiro Hiraoka, Kiyotaka Kawai, Tatsuya Harada, and Tomonori Ishikawa

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Learning Objectives

- To review the current techniques for intracytoplasmic sperm injection (ICSI) in human oocytes
- To clarify the problems in the current ICSI technique (Conventional-ICSI)
- To introduce a new technology of ICSI (Piezo-ICSI) to improve the survival and fertilization rates for injected oocytes
- To survey how Piezo-ICSI can contribute to the human assisted reproductive technology field

Key Points

- In the Conventional-ICSI technique, the cytoplasm is aspirated into the micropipette to break the membrane.
- The volume of cytoplasm aspirated into the micropipette at the membrane breakage point affects the fertilization rate after ICSI.
- In the Piezo-ICSI technique, the cytoplasm is not aspirated into the micropipette.
- Piezo-ICSI results in higher survival and fertilization rates than Conventional-ICSI.
- The micropipette wall thickness used for Piezo-ICSI affects the survival and fertilization rates after ICSI.
- Piezo-ICSI can contribute to shortening the training period for ICSI for junior embryologists.

39.1 Introduction

39.1.1 Brief History of Intracytoplasmic Sperm Injection (ICSI)

The first four pregnancies achieved by ICSI were reported by Palermo in 1992 [1], and ICSI is now an essential technique in human assisted reproductive technology (ART). ICSI uses beveled and spiked micropipettes (Fig. 39.1a) for mechanical penetration of the zona pellucida and the membrane as well as aspiration of the cytoplasm into the micropipette to break the membrane. After membrane breakage, the sperm is injected into the cytoplasm (Conventional-ICSI). However, the survival rate of mouse oocytes (oocyte diameter 80 μm) was as low as 16% (8% fertilization rate) after Conventional-ICSI [2].

Kimura and Yanagimachi performed membrane breakage by applying a piezo pulse, which produced ultrafast submicron forward momentum using uniquely shaped flat-tipped micropipettes with no bevel or spike (Fig. 39.1b) (Piezo-ICSI), in 1995 for mouse oocytes [2]. The survival rate of mouse oocytes was dramatically improved to 80% (78% fertilization rate) by using Piezo-ICSI [2]. Therefore, the Piezo-ICSI may be a less invasive method also for human oocytes (oocyte diameter 160 μm). However, to the best of our knowledge, only four reports detail the application of Piezo-ICSI to human oocytes, and little information is available regarding its clinical efficiency [3–6].

The goal of this chapter is to compare Conventional-ICSI and Piezo-ICSI techniques and to show the superiority of the Piezo-ICSI technique.

39.1.1.1 Conventional-ICSI Method

We used commercially available ICSI micropipettes with a beveled and spiked tip (Fig. 39.1a) (K-MPIP-1035, Cook Ireland Ltd., Ireland). The micropipette inner diameter was 5 μm, and the wall thickness was 1 μm. The micropipette was connected to a pneumatic injector (IM-9C, NARISHIGE Inc., Japan). The micropipette preparation was as follows. First, HEPES-buffered medium (SYDNEY IVF GAMETE BUFFER, Cook Australia Pty Ltd., Australia) was aspirated into the micropipette by capillary action for 1 min. Next, 7% polyvinylpyrrolidone (PVP) (7% PVP solution, Irvine Scientific, USA) was aspirated via negative pressure using an air injector. A motile sperm was immobilized by crushing the tail with the micropipette tip and aspirated tail-first into the micropipette in a 10 μl drop of 7% PVP. With the polar body at 12 o'clock, the micropipette was inserted through the zona pellucida into the oocyte (~90% of the oocyte diameter) to stretch the membrane (Fig. 39.2a–c). The membrane breakage procedure was performed as follows. Air was aspirated into the micropipette using an air injector to create negative pressure and suction on the membrane. The membrane was slowly aspirated into the micropipette (Fig. 39.2d) until a sudden flow of cytoplasm into the micropipette occurred (Fig. 39.2e), which was considered to be the moment of membrane breakage. After membrane breakage, positive air pressure was quickly provided to stop the flow of cytoplasm into the micropipette, and the sperm was injected into the oocyte (Fig. 39.2f).

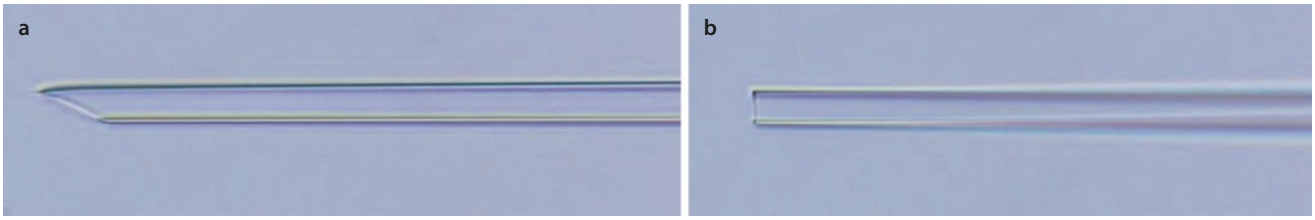

Fig. 39.1 Micropipettes for Conventional-ICSI **a** and Piezo-ICSI **b**

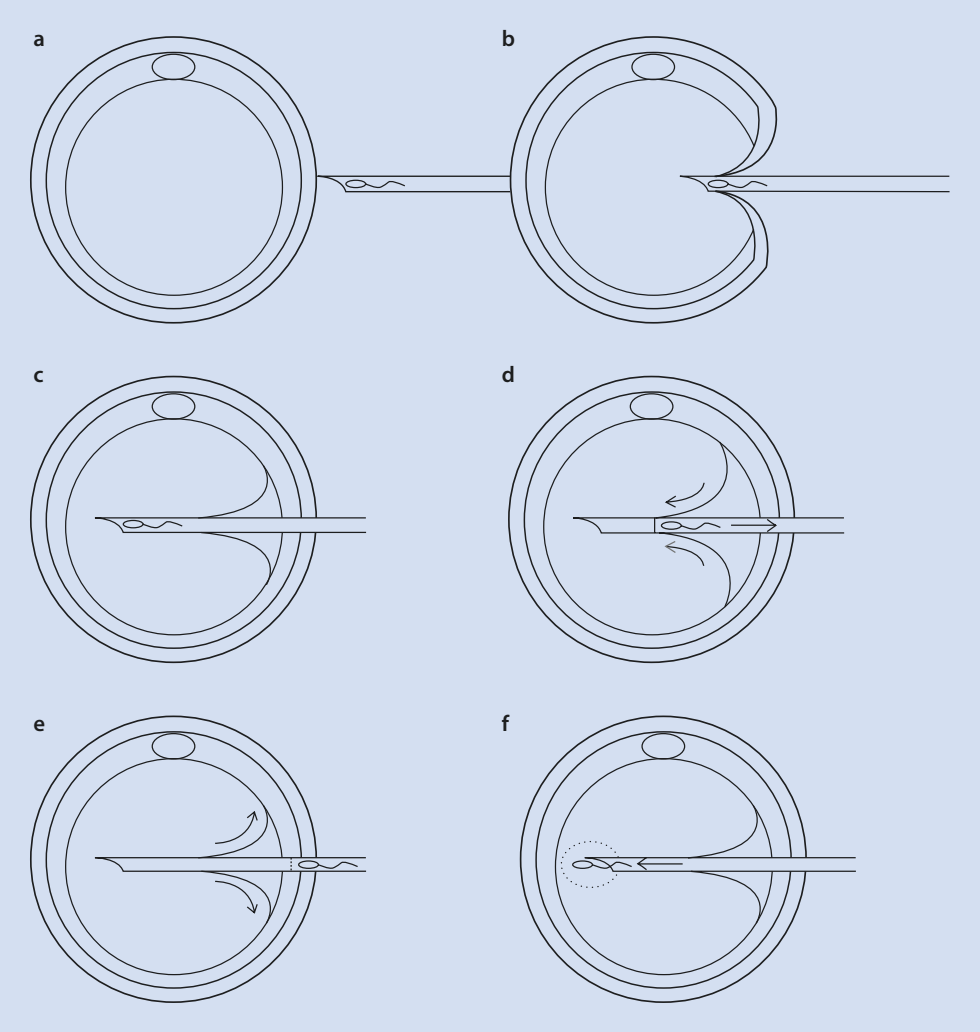

Fig. 39.2 Conventional-ICSI Before zona drilling **a**, during zona drilling **b**, after zona drilling **c**, during cytoplasm aspirating into the micropipette **d**, membrane breakage **e,** and sperm injection **f**

39.1.1.2 Piezo-ICSI Method

Characteristics of Piezo-ICSI

In Piezo-ICSI, membrane breakage is performed by applying a piezo pulse that produces ultrafast submicron forward momentum using uniquely shaped flat-tipped micropipettes with no bevel or spike (Piezo-ICSI) (Fig. 39.1b) [2]. During zona penetration, the injection pipette can penetrate the zona without zona or oocyte deformation, and during membrane breakage, no cytoplasm is aspirated into the micropipette.

Procedure for Piezo-ICSI

We used commercially available Piezo-ICSI micropipettes with a flat tip (PIN07-20FT, PRIME TECH Ltd., Japan). Fluorinert (6.25 µl, FC-770, 3 M) was aspirated to the middle of the micropipette (Fig. 39.3). Fluorinert is a clear, colorless, fully fluorinated liquid, which is nontoxic and water insoluble. The micropipette was inserted and clamped into the micropipette holder, which was then connected to the oil injector (HDJ-M3, PRIME TECH Ltd.). The piezo-micromanipulator drive unit (MB-S, PRIME TECH Ltd.) was attached to the micropipette holder

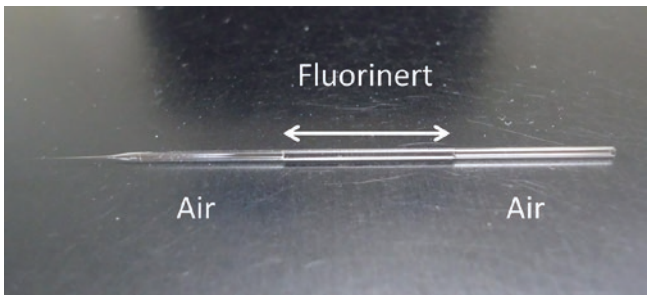

Fig. 39.3 Fluorinert placed in the middle of the micropipette for Piezo-ICSI

(Fig. 39.4). The piezo drive unit was driven by a controller (PMAS-ET150, PRIME TECH Ltd.). After Fluorinert was pushed to the micropipette tip, 6–12 pl of 7% PVP was aspirated into the micropipette. The sperm was then immobilized, as was done for Conventional-ICSI, and aspirated tail-first into the micropipette. Without oocyte deformation, the micropipette was placed gently against the zona pellucida while piezo pulses were applied to allow the pipette to break through the zona pellucida and not the membrane (Fig. 39.5a–c). The broken piece of the zona was expelled, and the sperm was advanced

Fig. 39.4 Piezo-micromanipulator drive unit for Piezo-ICSI

until the sperm head was near the micropipette tip (Fig. 39.5d). The micropipette was advanced forward (to ~90% of the oocyte diameter) to stretch the membrane (Fig. 39.5e, f). The membrane break was performed by applying one piezo pulse without aspirating the cytoplasm into the micropipette (Fig. 39.5g), and the sperm was injected into the oocyte (Fig. 39.5h).

Mechanism of Piezo-ICSI

Figure 39.6 shows the mechanism of zona pellucida opening or membrane breakage by piezo pulse. The Fluorinert is placed in the middle of the micropipette (Fig. 39.6a). The piezo pulse is applied producing an ultrafast submicron

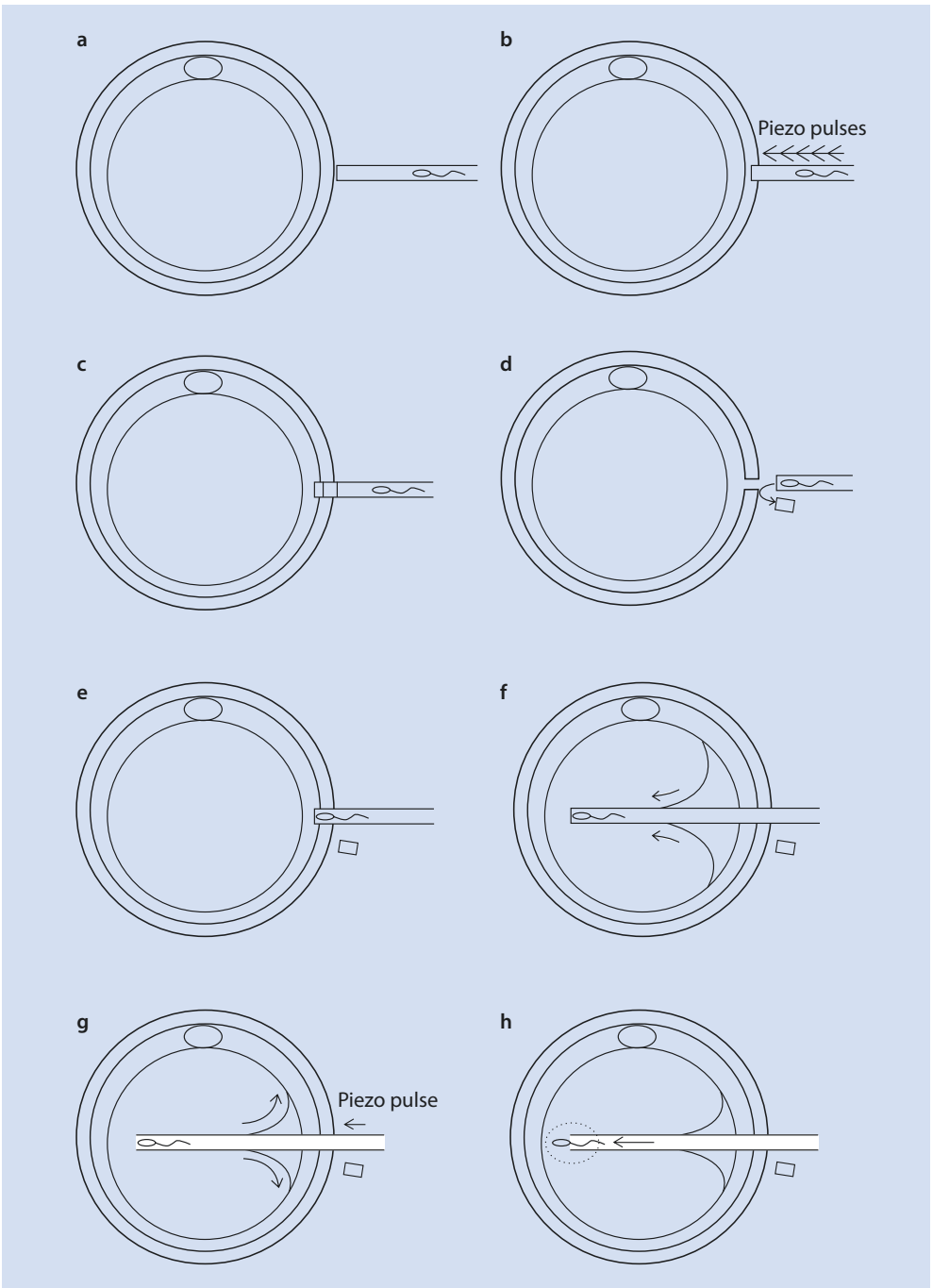

Fig. 39.5 Piezo-ICSI Before zona drilling **a**, during zona drilling **b**, after zona drilling **c**, expelling the broken piece of the zona **d**, during insertion of the micropipette tip through the drilled hole **e**, during stretching the membrane **f**, membrane breakage by applying the piezo pulse **g,** and sperm injection **h**

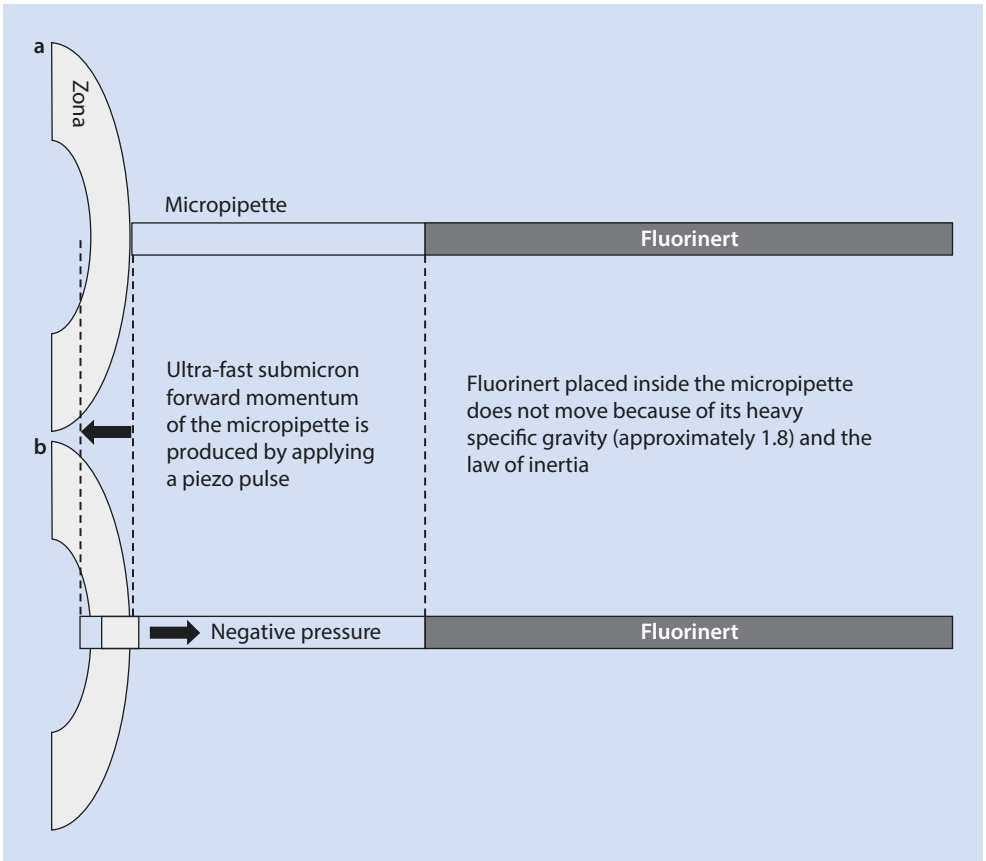

Fig. 39.6 Mechanism of Piezo-ICSI Before applying a piezo pulse **a** and during applying a piezo pulse **b**

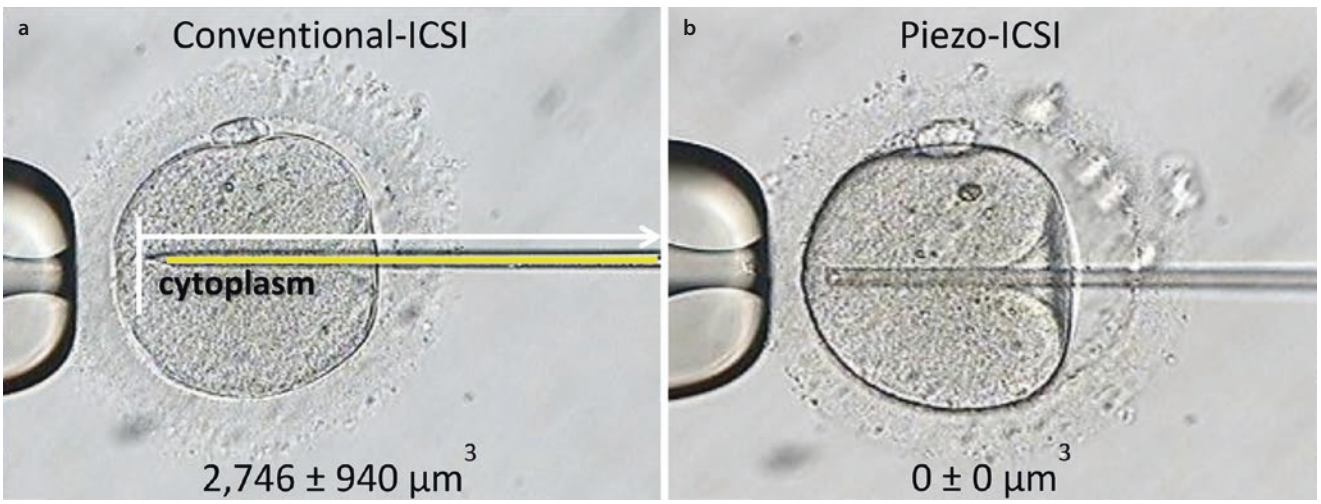

Fig. 39.7 Mean volume of cytoplasm aspirated into the micropipette at the point of membrane breakage from Conventional-ICSI **a** and Piezo-ICSI **b**

forward momentum of the micropipette. By applying a piezo pulse, only the micropipette is advanced forward, whereas the Fluorinert inside the micropipette does not move because of its heavy specific gravity (approximately 1.8) and the law of inertia. As a result, ultrafast submicron negative pressure is produced inside the micropipette tip. This ultrafast submicron negative pressure produces ultrafast submicron aspiration at the micropipette tip. This ultrafast aspiration induces the opening of the zona pellucida or membrane breakage (Fig. 39.6b). However, if the power of the piezo pulse is stronger, the zona or membrane can be destroyed, so the mechanism of Piezo-ICSI is still unclear.

39.1.1.3 Comparison Between Conventional-ICSI and Piezo-ICSI

In our previous analysis of 1341 oocytes from 286 patients, the calculated mean volume of cytoplasm aspirated into the micropipette with Conventional-ICSI (2746 ± 940 µm^3) (Fig. 39.7a)

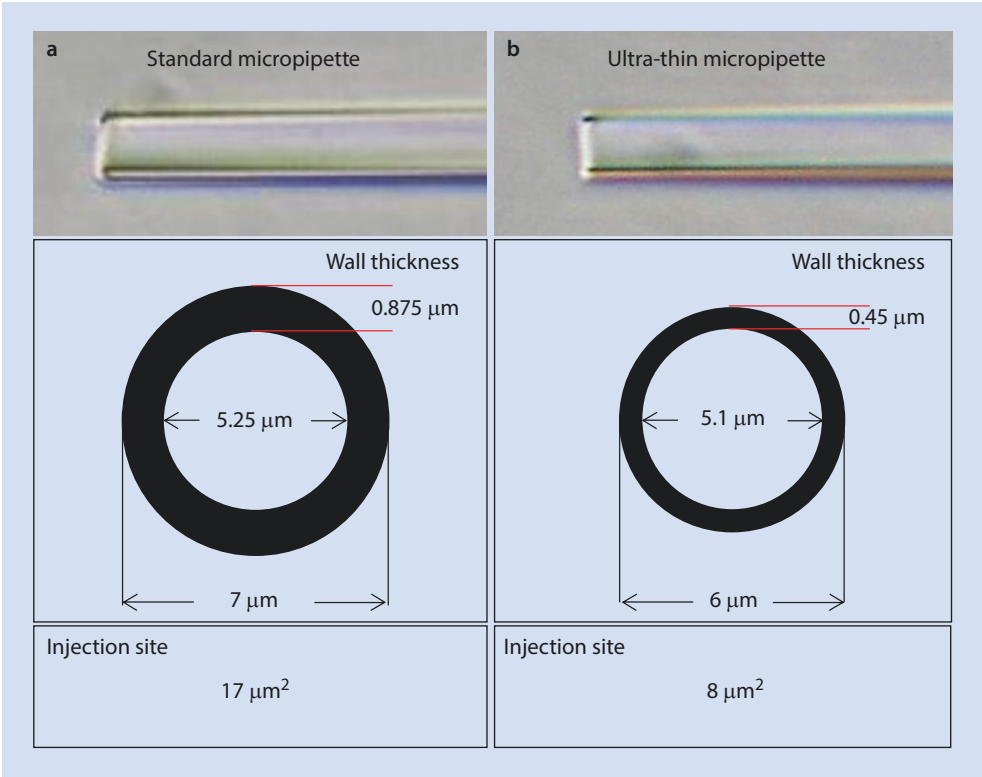

Fig. 39.8 Wall thickness, inside diameter, and injection site in the membrane for a standard micropipette **a** and an ultrathin micropipette **b**

was significantly higher than with Piezo-ICSI (0 ± 0 μm^3) (Fig. 39.7b) ($P < 0.05$) [6]. In addition, significantly higher survival and fertilization rates were observed when using Piezo-ICSI (717 oocytes from 166 patients) compared to Conventional-ICSI (624 oocytes from 120 patients) (survival rates 95% vs. 90%, fertilization rates 75% vs. 68%) ($P < 0.05$) [6]. When using the Conventional-ICSI method, the injection site in the membrane was larger due to the procedure of aspirating the cytoplasm into the micropipette during membrane breakage, which is avoided when using Piezo-ICSI. Moreover, Conventional-ICSI might also increase physical damage to the oocyte. As a result, the survival and fertilization rates using Conventional-ICSI were significantly lower than when using Piezo-ICSI. However, no significant differences were observed in embryo quality and pregnancy, implantation, or live birth rates between Conventional-ICSI and Piezo-ICSI. These results suggest that Piezo-ICSI can increase survival and fertilization rates without detrimental effects on embryo quality, implantation ability, or live birth potential.

39.1.1.4 Improvement in the Piezo-ICSI Technique

Standard Micropipette Versus Ultrathin Micropipette

We evaluated two micropipette wall thicknesses to determine the effect on the rates of survival, fertilization, good-quality day 3 embryos, pregnancy, implantation, and live birth during Piezo-ICSI. The standard micropipette had a wall thickness of 0.875 μm (Fig. 39.8a), and the ultrathin micropipette had a wall thickness of 0.45 μm (Fig. 39.8b). The membrane injection sites for the standard micropipette and ultrathin micropipette were 17 μm^2 and 8 μm^2, respectively (Fig. 39.8a, b) [6]. In our previous analysis of 1396 oocytes from 317 patients, significantly higher rates for survival (99% vs. 95%), fertilization (89% vs. 75%), good-quality day 3 embryos (55% vs. 43%), pregnancy (31% vs. 21%), implantation (31% vs.21%), and live births (25% vs.16%) were obtained when using the ultrathin micropipette (679 oocytes from 151 patients) than when using the standard micropipette (717 oocytes from 166 patients) for Piezo-ICSI ($P < 0.05$) [6]. The physical damage to the oocyte was reduced by creating a smaller injection site (17 μm^2 vs. 8 μm^2), which could partially explain the increased survival and fertilization rates. Consequently, we suggest that the combination of Piezo-ICSI and the ultrathin micropipette can significantly improve the effective utilization rate of injected oocytes and can increase live birth rates.

Oil Injector Versus Air Injector

In preparation for Piezo-ICSI, we aspirated approximately 1–2 cm of Fluorinert to the middle of the micropipette (Fig. 39.3). Next, this micropipette was inserted into the micropipette holder of the oil injector filled with mineral oil. The mineral oil flows inside the micropipette, pushing the air and Fluorinert forward to the micropipette tip. Figure 39.9a shows a good example of micropipette preparation. However, if air bubbles occur in the mineral oil (Fig. 39.9b) while inserting the micropipette into the micropipette holder, Piezo-ICSI does not work. A hole does not open in the zona, and the membrane does not break. In this case, this micropipette is discarded. Because

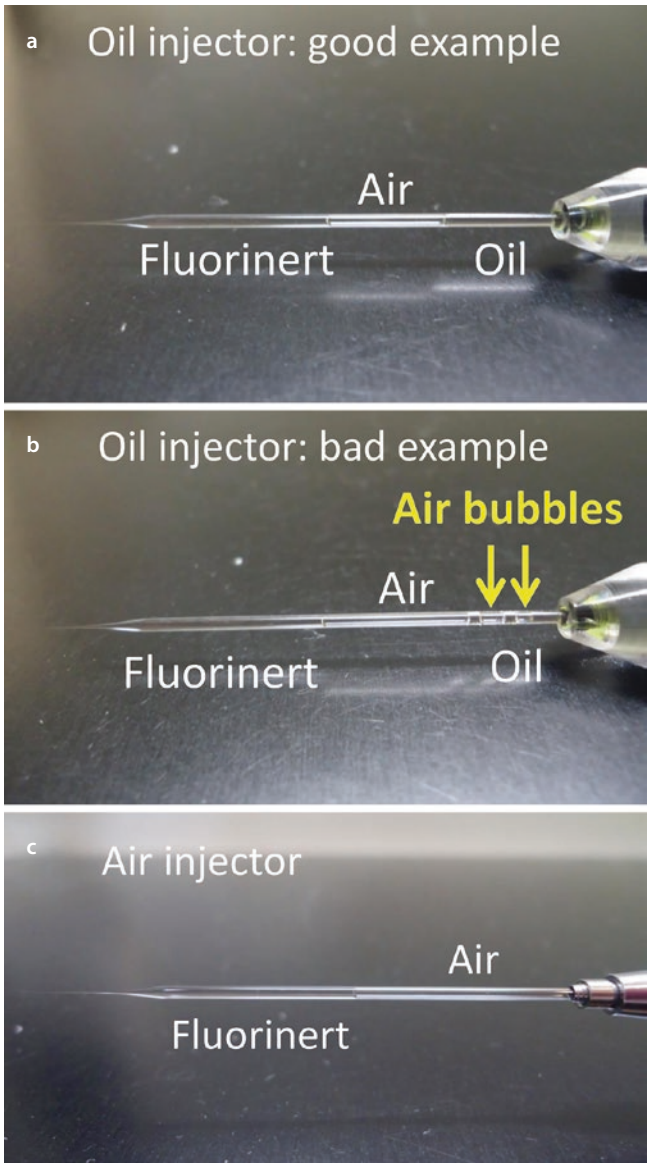

Fig. 39.9 Preparation of Piezo-ICSI using an oil injector (**a**: good example, **b**: bad example) and air injector **c**

the oil injector is sticky from the mineral oil, micropipette preparation takes time. Therefore, if an air injector for Piezo-ICSI can be used, the number of wasted micropipettes and preparation time should be reduced. However, little information is available regarding the clinical efficacy of Piezo-ICSI with an air injector. Therefore, we assessed the clinical efficiency and safety of Piezo-ICSI with an air injector. In our previous analysis of 780 oocytes from 180 patients, we measured the time for micropipette preparation with the oil injector (409 oocytes from 90 patients) and air injector (371 oocytes from 90 patients). The average time for the oil injector was 233 s, whereas the average time was significantly reduced to 106 s with the air injector ($P < 0.05$) [7]. We also counted the number of wasted micropipettes due to air bubbles. The average number of wasted micropipettes from the oil injector per patient was 0.28 ± 0.56. However, when using the air injector, which was free from mineral oil and thus air bubbles were not possible (Fig. 39.9c), no micropipettes were wasted [7]. No significant differences were found between the oil and air injectors in the survival (99% vs. 99%), fertilization (89% vs. 90%), or good-quality day 3 embryo (61% vs. 61%) rates [7]. During this study, an extra 3.2 h and 25 micropipettes were used for the oil injector group (90 patients) compared to the air injector group (90 patients) [7]. Our results indicate that the air injector dramatically reduced the waste of time and micropipettes occurring with the oil injector and did not impair the survival, fertilization, or good-quality day 3 embryo rates. Therefore, Piezo-ICSI with an air injector is clinically efficient and safe.

Head-First Injection Versus Tail-First Injection

In human oocyte ICSI, a sperm is injected head-first into the cytoplasm during fertilization because sperm internalization into the cytoplasm is initiated from the sperm head in natural fertilization. However, ICSI procedures bypass hyperactivation, zona pellucida penetration, and internalization of the sperm head into the cytoplasm. Because the sperms are injected directly into the cytoplasm, the oocytes could be fertilized if injected tail-first. However, little information is available regarding the effect of the sperm direction during injection into the cytoplasm for Piezo-ICSI results and embryo development. In order to inject sperm head-first into the cytoplasm, the sperm is aspirated from the tail into the micropipette. This procedure is technically difficult owing to the small tail size. Head-first sperm aspiration into the micropipette would be easier and faster. Therefore, we assessed the effects of sperm direction (head-first or tail-first) during injection into the cytoplasm on oocyte survival, fertilization, and embryo development. For head-first injection, the sperm was aspirated into the micropipette tail-first and injected into the oocyte head-first (Fig. 39.10a); for tail-first injection, the sperm was aspirated into the micropipette head-first and injected into the oocyte tail-first (Fig. 39.10b). In our previous analysis of 632 oocytes from 152 patients, we calculated the duration of sperm manipulation (from starting sperm immobilization to aspiration of the sperm into the micropipette). The average time for sperm manipulation during head-first injections (342 oocytes from 75 patients) was 10.5 ± 1.6 s, whereas with the tail-first injections (290 oocytes from 77 patients), the time was significantly reduced to 8.6 ± 1.8 s ($P < 0.05$) [8]. No significant difference was found between the head- and tail-first injections in the survival rates (99% vs. 99%), fertilization rates (86% vs. 90%), or good-quality day 3 embryo rates (69% vs. 68%) [8]. Our results indicate that the sperm direction during cytoplasmic injection does not affect oocyte survival, fertilization, and subsequent embryo development (good-quality day 3 embryo rate) with Piezo-ICSI. However, aspiration of sperm into the micropipette is easier and faster using sperm tail-first instead of head-first. Consequently, we recommend injecting the sperm into the cytoplasm tail-first during Piezo-ICSI.

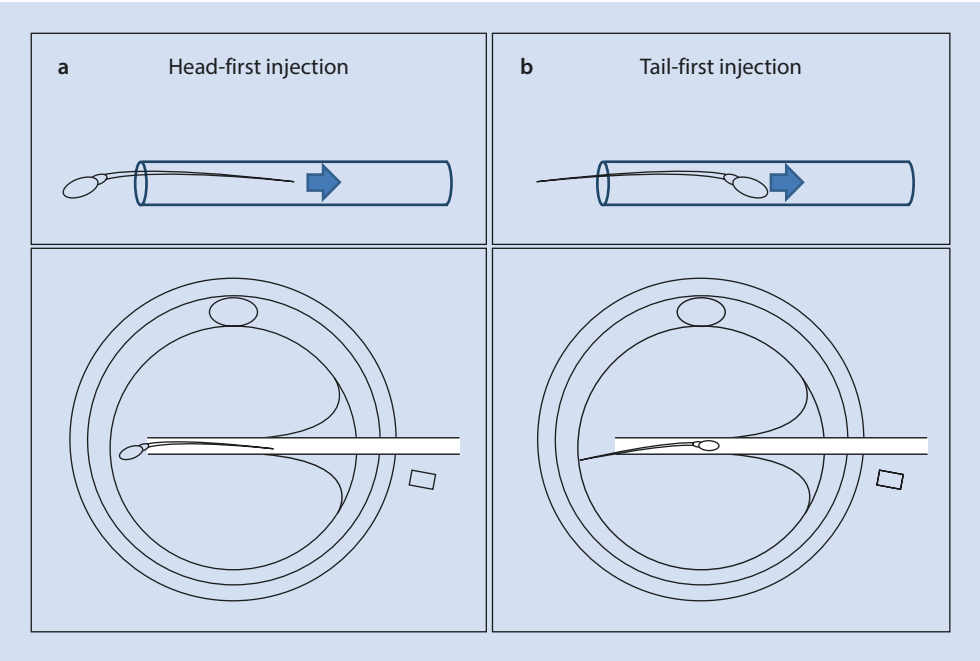

Fig. 39.10 Head-first injection **a** and tail-first injection **b** during Piezo-ICSI

39.1.1.5 Advantages of Piezo-ICSI

Improvement in Fertilization Rate and Reduced ICSI Training Period

The fertilization rates for Conventional-ICSI published in the 2000s were 62–77% [9–13]. Similarly, the fertilization rate for Conventional-ICSI performed by three junior embryologists at our hospital, Kameda Medical Center (Kamogawa City, Chiba, Japan), was 66%. We sought to improve this fertilization rate using Piezo-ICSI. However, little information was available regarding if Piezo-ICSI improves the fertilization rate for procedures performed by junior embryologists and, if so, how many procedures are needed to improve the rate. We assessed whether introduction of Piezo-ICSI could improve the fertilization rates and, if so, the number of procedures needed to see improvement. The study subjects were three junior embryologists. They had performed Conventional-ICSI for 5, 5, and 1 year, respectively. They received Piezo-ICSI training from a senior embryologist who had performed Conventional-ICSI for 11 years and Piezo-ICSI for 4 years. The fertilization rate for the procedures performed by the senior embryologist at Kameda Medical Center was 83%. The fertilization rate for the senior embryologist per 20 oocytes (120 oocytes in total) was more than 80%. Thus, we considered our junior embryologists proficient in performing Piezo-ICSI when their fertilization rate improved to ≥80% per 20 oocytes. In our previous analysis of 1373 oocytes, the fertilization rate for Conventional-ICSI (between February 2014 and September 2014) performed by the three junior embryologists was 66%, whereas the fertilization rate with Piezo-ICSI (between October 2014 and June 2015) significantly improved to 82% ($P < 0.05$) (Fig. 39.11). The fertilization rates for Conventional-ICSI performed by junior embryologists I, II, and III were 60%, 74%, and 64%, respectively. The fertilization rates for Piezo-ICSI performed by junior embryologists I, II, and III were 80%, 83%, and 83%, respectively. The fertilization rates in the case of each junior embryologist using Piezo-ICSI were significantly higher than that for Conventional-ICSI ($P < 0.05$). After 20 procedures, the fertilization rates from the junior embryologists using Piezo-ICSI reached ≥80% per 20 oocytes (unpublished data). Our results indicate that Piezo-ICSI significantly improved the fertilization rates for the procedures performed by three junior embryologists from 66% to 82%, and they became proficient after 20 procedures.

39.2 Summary

The rates of survival and fertilization using Piezo-ICSI with standard micropipettes in our previous results (survival rate 95%, fertilization rate 75%) are not superior to those recently reported using Conventional-ICSI (survival rate 89–93%, fertilization rate 62–77%) [9–13], suggesting that Piezo-ICSI with a standard micropipette may not be optimized for human oocytes. However, Piezo-ICSI with ultrathin micropipettes resulted in significantly higher survival and fertilization rates than with standard micropipettes (survival rates 99% vs. 95%, fertilization rates 89% vs. 75%) [6]. Furthermore, as shown in this chapter, we designed a new Piezo-ICSI methodology using an air injector and tail-first injection for a more user-friendly Piezo-ICSI technique. By using this modified Piezo-ICSI, we significantly improved the ICSI fertilization rate performed by our junior embryologists from 66% to 82% after 20 procedures. We believe that Piezo-ICSI can not only improve the ICSI fertilization rate but also shorten the training period for ICSI practitioners. Further study is needed to assess whether Piezo-ICSI can improve the fertilization rate and shorten the training period for ICSI at other ART institutions.

Fig. 39.11 Monthly and total fertilization rates of Conventional-ICSI (between February 2014 and September 2014) and Piezo-ICSI (between October 2014 and June 2015) for three junior embryologists at Kameda Medical Center

Review Questions

1. What kind of characteristic does the current ICSI technique (Conventional-ICSI) have during membrane breakage?
2. What kind of characteristic does a new technology of ICSI (Piezo-ICSI) have during membrane breakage?
3. How can Piezo-ICSI contribute to the training period for ICSI for junior embryologists?

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Embryo Evaluation, Grading, and Assisted Hatching

Contents

Traditional Embryo Morphology Evaluation: From the Zygote to the Blastocyst Stage

Jonathan Kort and Barry Behr

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Learning Objectives

- Understand the importance and limitations of morphological assessments at each key stage of preimplantation embryo development.
- Understand methods for fertilization check and apply pronuclear scoring systems.
- Understand methods for cleavage-stage embryo assessments, including assessments of multinucleation, cell number, and fragmentation.
- Apply grading systems for blastocyst evaluation.

40.1 Introduction

After in vitro fertilization, providers are repeatedly tasked with assessing embryos at various stages in order to make critical, time-sensitive patient care decisions at each stage of embryo culture using morphological assessments. At the zygote stage, assessment of normal fertilization must occur just before the brief window in which pronuclei are visible closes in order to determine which embryos are suitable to continue in culture. Cleavage-stage assessments must determine whether a patient should transfer early or pursue blastocyst culture before transfer or cryopreservation. Finally, blastocyst morphology assessments must be made to determine if and when embryos are optimally ready for trophectoderm biopsy for cases undergoing genetic screening, if and when embryos are optimally ready for cryopreservation, and, most importantly, which embryos are most likely to become a healthy pregnancy when selecting an embryo for transfer. To compensate for imperfect embryo selection methods, clinicians in the US transfer multiple embryos in 67–89% of cases [1] leading inevitably to multiple gestation pregnancies and their inherently high risk of prematurity and other complications [2]. Due to health, legal, and financial consequences of multiple gestation pregnancies, single embryo transfer remains the ideal for IVF patients and providers and, in some countries, has already been mandated for these reasons with a consequent reduction in multiple gestation pregnancies and perinatal morbidity [3].While molecular and biochemical methods to assess human gamete and embryo reproductive potential have been described and are increasingly utilized, these methods are still relatively complex, time-consuming, and expensive for most ART laboratories. Hence, the assessment of morphology has been, and will remain, the most widely employed method for embryo selection as it is quick and inexpensive and, although not without faults, has consistently been shown to have predictive value.

Most morphological methods of embryo assessment rely on a single static observation of the embryos, whereas the embryo itself, after fertilization, is on a precise set of clocks governing the division and initiation of key events from gene activation through compaction and blastulation. Each stage of the complex developmental process is dependent upon the successful completion of the previous one. Consequently, the optimal evaluation of an embryo's potential would require multiple, dynamic assessments of the embryo at each developmental checkpoint as it makes its complex transition from a single-celled zygote to a blastocyst. A comprehensive morphological evaluation should assess the end result at each checkpoint but also the path taken to reach that point. An embryo that does not meet a selection criterion at one point may meet it at another but perhaps should be avoided or at least flagged and deprioritized, as early deviation from optimal development may indicate inherent abnormalities within the embryo. Performing assessments in a systematic, cumulative fashion should provide the most predictive information regarding embryo quality. This chapter will outline the various morphological and morphokinetic criteria that are currently available for selection within the framework of a continuous model from the zygote through to the blastocyst. No one selection criterion is better, but if possible a combination of one, two, or even three selection points should lead to the most accurate selection [4].

Apparent in this chapter will be arguments in the literature regarding the utility of particular morphological assessments. The lack of standardization regarding assessment timing, techniques employed in culture such as hatching, and heterogeneous inclusion criteria among the literature make it difficult to achieve consensus regarding the application of many of the methods described. Additionally, given new research regarding the importance of synchrony between the embryo and endometrium and the effect of ovarian hyperstimulation on endometrial receptivity [5, 6], many of the ideal morphological characteristics that were determined in studies utilizing fresh embryo transfers will need to be revisited in studies employing frozen embryo transfers to ensure characteristics deemed unfavorable are not merely out-of-sync in a fresh cycle rather than reflective of reduced embryo viability.

40.2 Zygote Quality Day 1: Pronuclear Scoring Systems

The fertilization check, occurring 16–18 hours after conventional insemination or intra-cytoplasmic sperm injection (ICSI), offers the opportunity to confirm normal fertilization by the presence of two pronuclei but also subjectively assess the pronuclei using any of a number of systems designed to offer insight into the overall viability of the embryo based on the symmetry, position, and number of pronuclei and nucleoli [7]. All pronuclear scoring systems discussed here [8–11] attempt to classify zygotes based on the following characteristics of the two pronuclei: symmetry (equal vs. unequal), position (in apposition vs. at a distance), and location (central vs. noncentral). The nucleoli are scored based on number [3–7], symmetry (equal vs. unequal sizes), and location (polarized or aligned vs. nonpolarized or nonaligned).

At the fertilization check, visualization of the pronuclei should be attempted using an inverted microscope with Hoffman modulation optics [12]. Zygotes should be rolled during observation and positioned with simultaneous visualization of the polar bodies and pronuclei if present. While normal fertilization is traditionally confirmed by the presence of two pronuclei, it has been demonstrated that healthy babies can develop from monopronuclear [13] or even zero-pronuclear zygotes [14]. While primarily thought to indicate parthenogenetic activation of the oocyte, a single pronuclei may actually only demonstrate the asynchrony in pronuclei formation, which has been observed in over one-third of zygotes [15] and more commonly after conventional insemination than ICSI [16] or result from pronuclear fusion [17] during syngamy and should be continued in culture, particularly after conventional insemination, in case no 2-pronuclei derived embryos are available for transfer. Similarly, 0-pronuclei zygotes may actually represent normally fertilized embryos with an accelerated cell cycle and breakdown of pronuclear membranes with no visible pronuclei at the time of fertilization check [18]. These embryos can also be continued in culture for selection if no 2-pronuclei embryos are available. Zygotes with >2 pronuclei are a result of polyspermy, usually after IVF, in which there are three or more pronuclei but also two polar bodies or failure to extrude the second polar body in which there are three pronuclei and one polar body (digynic) [19]. After IVF ≥3 pronuclei is indicative of a high incidence of mosaicism, and after ICSI digyny conveys a high incidence of polyploidy. Due to the limitations of most molecular diagnostic methods of preimplantation genetic screening not being capable to detect polyploidy, zygotes with ≥3 pronuclei should not be continued in culture, although healthy offspring have been reported without verification by neonatal karyotyping [20].

For zygotes with 2-pronuclei at the time of transfer, pronuclei scoring systems can potentially be employed. The initial pronuclei scoring of Scott et al. was based on parameters such as pronuclear size and alignment, alignment of nucleoli within the pronuclei, and cytoplasmic morphology such as appearance of cytoplasm, presence of a halo, timing of nuclear membrane breakdown, and early cell division [8]. The total score ranged from 7 to 25 with an optimal score ≥15. Their revised score included only parameters that could be assessed at the time of fertilization assessment (17–18 hours post-insemination), classifying the zygotes by five characteristics including the presence of a halo, the orientation of the nuclei relative to the polar bodies, and the size, number and pattern of distribution of nuclear precursor bodies (NPB) in the nuclei. According to this system, the ideal pronuclei should be symmetric and centrally located aligned onto the polar axis for the completion of mitotic division. Additionally, the intracellular movement required to achieve this alignment results in the favorably observed halo. NPBs are ideally equal in number and size and aligned between the two pronuclei although nonalignment is preferable to asymmetry. Scott's group demonstrated that employing a composite pronuclei score, denoted as the Z-score, significantly increased pregnancy and implantation rates during cleavage stage and blastocyst transfers [8, 10]. Tesarik and Greco also claimed pronuclei assessment could predict embryo development; however, they focused their assessment on the number, distribution, and synchrony of NPB progression within each pronucleus [11, 21]. These authors considered interpronuclear synchrony, evaluated at 12–20 hours after IVI/ICSI, to be more important than the actual NPB polarity at the site of pronuclear apposition as they assumed that polarization of nucleoli was a dynamic process and may not be evident from the beginning of pronuclei formation. Using this assessment system, Tesarik and Greco were able to retrospectively demonstrate that zygotes with more favorable NPB assessments had higher rates of embryo development with better morphology and higher implantation rates.

Despite initial promise for the predictive value of pronuclei assessment, subsequent studies have been inconsistent. Multiple other groups failed to demonstrate correlation between pronuclear assessments and pregnancy rates [15, 22–24]. Similarly, no consensus exists regarding the utility of using the presence of a halo as a marker of embryo potential with studies demonstrating its presence as having a positive [9, 22, 25, 26] effect, but another found that having an excessive halo was also a negative prognostic [27] for blastocyst development.

Zygote pronuclei assessments are limited by the static nature of assessments and dynamic nature of pronuclear morphology, a range of biological variation within normal development, particularly as a result of heterogeneous lab techniques such as ICSI [16] or oocyte cryopreservation and embryo characteristics [28]. Consequently, no clear consensus regarding the utility of zygote grading has been reached. Additionally, accurate assessment of the three-dimensional disposition and number of NPBs is technically difficult while avoiding prolonged exposure of the zygote to light, temperature, and pH shifts. With the advent of incubator time-lapse imaging systems with improved optics, this topic may be revisited in the near future.

40.3 Timing and Result of the Initial Cleavage

The LH surge, or trigger injection for patients undergoing IVF, starts the clock which dictates the timing of events as the oocyte progresses through the completion of meiosis [29]; however, many of the assessments performed in clinical labs are based on the timing of insemination or ICSI. With any time-based embryo assessment, it is critical to standardize the reference point from which the clock is started to ensure genuine comparisons. While the use of time-lapse imaging for embryo selection has received increasing attention more recently, researches have been interested in the timing of the first cell division as a prognostic marker for embryo viability for almost 20 years [30, 31], when it was noted that embryos undergoing "early cleavage" (<25 hours post-insemination) had higher implantation rates than those that did not.

Typically, embryos enter the 2-cell stage 20–36 hours post-insemination [32, 33]; however, numerous studies have demonstrated that embryos that undergo initial cleavage earlier in that spectrum have improved blastocyst formation rates, pregnancy rates, and even higher rates of euploidy [10, 30, 31, 34–41]. Again lab technique can significantly influence downstream events, as embryos fertilized by ICSI have been observed to undergo cleavage earlier than those undergoing conventional insemination [39], affirming the importance of standardizing comparisons whether clinically or for research. More recent studies employing time-lapse imaging have demonstrated the limitations of a single static assessment of a dynamic process, and it is likely our interpretation of the fluid events involved in early embryo development will be more refined with increasing use of this technology [33].

In addition to the timing of the initial cleavage, the result of the initial cleavage has important prognostic implications. In multiple time-lapse studies, embryos that proceed directly from the 1-cell stage to ≥3-cell stage have significantly reduced, if not nonexistent, implantation rates [42, 43] as do embryos with marked blastomere asymmetry at the 2-cell stage. These findings support the potential benefit of using dynamic morphological assessments (i.e., time-lapse imaging) to augment morphological embryo assessments and expose the limitations of single static assessments at each stage.

40.4 Cleavage-Stage Embryo Assessments

Most clinical IVF programs have now incorporated blastocyst culture to some extent into their practice. By culturing embryos beyond the cleavage stage, it identifies embryos without a development block at the cleavage stage identifying those with the greatest reproductive potential [44] and facilitates other embryo selection methods such as preimplantation genetic screening. Some question the utility of cleavage-stage assessments, particularly for patients committed to blastocyst culture. To the contrary, some studies suggest cleavage-stage enhanced blastocyst selection [45], and there are still sufficient poor prognosis patients with few embryos pursuing IVF that need to decide whether to transfer at cleavage stage or pursue blastocyst culture.

After evaluating transfers with high implantation rates, researchers have been able to develop multiple scoring systems to assess cleavage embryos on days 2 and 3. Most focus on the presence/absence of multinucleation, fragmentation, and blastomere number, size, and morphology as these parameters have been found to be most predictive of subsequent development and favorable transfer outcomes [46, 47].

40.5 Multinucleation

The presence of ≥2 interphase nuclei within a single blastomere qualifies an embryo to be multinucleated, a common characteristic present in at least one embryo in roughly 75% of IVF cycles [48, 49]. This phenomenon can be a result of chromosome segregation errors in the first cleavage. Mitotic replication without the first cytokinesis explains instances when multinucleated and sibling mononucleated blastomeres have the same genetic composition. Nuclei fragmentation into multiple nuclei would result in a different instance in which a multinucleated blastomere would have the sum total chromosomes as found in mononucleated sibling blastomeres. Finally, defective mitotic spindles and consequent segregation errors are theorized to explain instances where the number of a particular chromosome was double, triple, or quadruple the copy number of mononucleated cells, and the replicates of this same chromosome were randomly distributed among the multiple nuclei [32, 50]. Multinucleation can be identified on either day 2 or day 3 of embryo culture, although its prevalence may be higher on day 2. It is unclear to what extent this observation is due to optical factors including the larger dimensions of cells and less obstruction from fewer overlapping cells on day 2. Alternatively, this may be indicative of the biologically transient nature of multinucleation [48, 51] which may result from embryos' repair mechanisms [52] as blastocysts have been observed to have a far lower incidence of nuclear abnormalities than cleavage-stage embryos [53].

Multinucleation has been deemed an unfavorable characteristic due to its correlation with higher rates of embryo aneuploidy, impaired cleavage and development, and poorer implantation rates [46, 50, 53–59]. Some have proposed that stimulation-specific factors such as shorter duration of stimulation and use of higher gonadotropin doses influence the incidence of multinucleation [48], although others have attributed other factors such as lab environment [60], although many of these contributing factors and subsequent observations likely confound each other due to the high coincidence of multinucleation with poorer embryo quality, poorer patient population, and higher rates of fragmentation and lower cleavage rate and cell number. Better resolution imaging and innovative staining techniques will likely lead to additional investigation of the cleavage-stage nucleus and the association of morphologic abnormalities with embryo development and reproductive potential [53].

40.6 Cell Number

Many studies have reported observations of an optimal cleavage pattern, citing higher pregnancy rates after transfer of former day 2 4-cell embryos than those at the 2- or 3-cell stage [61–63]. Additionally, the path to the 4-cell stage appears to be as, if not more, important than the end result. The 2-cell embryo proceeds through an intermediate 3-cell stage before quickly cleaving again to form a 4-cell tetrahedron embryo [64, 65]. An embryo which is an even-sized 3-cell that is not progressing rapidly to the 3-cell stage is likely abnormal, demonstrating cytokinetic delays, which, in some studies, have been predictive of a zero live birth rate [66]. While delays in reaching the 4-cell stage have prognostic

value for poor subsequent development, Guerif and others have observed that too rapid cleavage (5–8 cells at day 2) was also predictive of lower blastocyst formation rates [67–69]. Possible hypotheses to explain this phenomenon include that some blastomeres may actually be large anucleate fragments or that accelerated early cell division may indicate developmental instabilities and have higher rates of aneuploidy [62, 70]. While Guerif did find a correlation between day 2 development and blastocyst formation, after controlling for blastocyst morphology and other confounders, they did not find day 2 morphology predictive of blastocyst implantation [67].

Day 3 blastomere number has also shown value in predicting subsequent development, albeit with a wider acceptable range of cell number. Various studies have shown a positive correlation of the number of cells in day 3 embryos (up to 8) with implantation rates following day 3 transfer [71, 72], rate of blastocyst formation [73], and pregnancy rates [73, 74] when compared to embryos with less than eight cells. Consistent with these findings, Alikani also observed that embryos with 7–9 cells on day 3 converted to blastocysts at a significantly higher rate than day 3 embryos with <7 cells or >9 cells [63], and further refining this ideal range, Racowsky et al. observed that embryos with exactly eight cells on day 3 had the highest implantation rates [75].

40.7 Fragmentation

Embryo fragmentation is a result of the expulsion of anucleate, membrane-bound cytoplasm that occurs during embryo cleavage and is associated with aberrant cell division [32, 71, 76, 77]. These fragments have been argued to be both by-products and catalysts of apoptosis [78, 79] or an indicator of the irregular loss of synchrony between nuclear and cytoplasmic cell division. While it is difficult to demonstrate whether this is a consequence or precursor of fragmentation, surviving blastomeres may be depleted of critical organelles, substrates, or signal ligands [80–83] as a result of excessive fragmentation affecting future development and reproductive potential.

Assessment of fragmentation is typically quantified as the percentage of the embryo the fragments occupy [84], although the timing pattern of fragmentation has also been demonstrated to be associated with genetic abnormalities when applied with other morphokinetic parameters [85]. One scoring system simply assigns values based on the volume occupied by fragments (score 0 = 0%, score 1 = <10%, score 2 = 10–25%, score 3 = >25%) [86], while others have proposed a more detailed classification taking into account the size and location of fragments relative to the size and position of nucleated cells [76]. Regardless of the system used, increasing fragmentation on day 2 of culture has been associated with poorer blastocyst formation rates, possibly due to the association with genetic abnormalities [63, 67, 87–89]. It is unclear if the fragments are a cause or coincidental result with poorer outcomes. Some postulate that the fragments physically impede cell-cell interactions and interfere with blastulation, while others hypothesize the fragments release substances toxic to continued development [76, 90], supporting the case for microsurgical removal which has been performed with mixed results [91, 92].

More recently, with the advent of time-lapse imaging, fragment reabsorption and the presence of chromosomes within fragments have been demonstrated [32, 85] indicating that the interpretation of fragmentation may be more complex than simply its presence or absence. It is likely that fragmentation is the common end result of a heterogeneous multitude of events, which would explain why fragment or lysed blastomere removal had inconsistent results for improving clinical embryo culture outcomes [91–95].

40.8 Blastomere Symmetry and Appearance

Textbook mitosis results in an even number of cells with the cytoplasm equally split between daughter cells, resulting in even numbers of similar-sized cells, which decrease in size with each round of cell division. This ideal is reflected in many cleavage-stage embryo grading systems which extrapolate that uneven distribution of organelles, proteins, and RNA parallel asymmetric division and may be detrimental to embryo development [72, 96, 97]. Despite this concept, slight asymmetry at a random static observation is normal in human development that results in transient 3-, 5-, and 7-cell embryos to allow for cell spacing as the embryo divides [65]. Though more studies are needed to investigate the link between cell asymmetry and embryo development, studies have demonstrated that asymmetry is predictive of poor subsequent development [57, 98, 99], reduced implantation rates [61, 98], and higher rates of aneuploidy [38, 100, 101]. Day 2 cell asymmetry, regardless of morphology on days 1, 3, and 5, precluded live birth and markedly reduced the rate of a clinical pregnancy in one study, highlighting the biological importance of cell symmetry at the early cleavage stage [102]. Less consistently, blastomere cell size has been linked to the extent of fragmentation although other studies have not uniformly validated this finding [97, 99, 102].

40.9 Day 4 Assessment

Due to the rarity of transfers occurring on day 4, day 4 embryos are rarely assessed in clinical practice [103] despite the potential prognostic value of the relative timing of compaction and morulae formation [104, 105]. Compaction occurs simultaneously with the development of cell polarity. As blastomeres flatten against neighbors, cell-to-cell adhesion transitions to gap and tight junctions resulting in the loss of discernable cell borders, and cytoplasmic polarization begins to appear as a result of embryo transcription. Consequently, the blastomeres arising from the next cleavage segregate to inside apolar and outside polar cells, which may be largely determined by their pre-differentiation position within the

embryo [106]. Compaction typically occurs on day 4, while early and late compaction is regularly observed in culture. Early compaction on day 3 has been shown to positively predict implantation in embryos with minimal fragmentation, although surprisingly it negatively correlated with implantation in cleavage-stage embryos with >10% fragmentation [104]. This paradoxical result may reflect inadequate stratification in the study. Others have created more comprehensive day 4 embryo scoring systems which include fragmentation and vacuolization in addition to compaction and have reported equivalent embryo selection capabilities to blastocyst selection methods for single embryo transfers [107]. While there may be prognostic value in day 4 assessments, given the increasing utilization of trophectoderm biopsy for genetic screening and the informative variety of morphology observed in day 5/6 development, the demand for blastocyst culture and transfer will likely outweigh the utility of day 4 assessments for the foreseeable future.

40.10 Blastocyst Assessment

The blastocyst represents the ultimate stage of clinical embryo culture before transfer back into the uterus, and therefore it is the final stage at which morphological assessment is possible. Culturing to the blastocyst stage helps reveal which cleavage-stage embryos have developmental blocks and should not be transferred and offers an opportunity to make assessments at the closest point possible prior to implantation. Some studies report that blastocyst morphology assessments select different embryos for transfer than day-3 embryo morphology assessments in at least half of all cases [108–110]. Consequently, extending culture until the blastocyst stage on day 5 or 6 is likely beneficial for embryo selection, although some patients will not have any blastocysts available for assessment and transfer.

The blastocyst is defined by the onset of cavitation and the formation of the blastocoel which coincides with cell differentiation between the inner cell mass (ICM) and trophectoderm (TE) [111]. These defining aspects of the blastocyst have become the focal points of morphological assessment systems which typically focus on the degree and timing of expansion, the quality of the trophectoderm, and the inner cell mass. The Gardner and Schoolcraft grading system and the similar system outlined in the Istanbul consensus document are the two most common blastocyst grading frameworks used and focus on these aspects [112–114]. A weakness of any static morphological assessment is that the blastocyst is very dynamic, and even a short interval of minutes can change the assessment significantly. Embryos should be assessed approximately 154 hours after hCG or 112–114 hours following insemination for blastocyst development [102].

A defining aspect of the blastocyst, generation of the fluid-filled blastocoel cavity, is a result of the Na/K-ATPase membrane channels which increase the salt concentration within the embryo, driving water into the cavity through osmosis. Due to the work of the pump, the size of the blastocoel expands, and ultimately hatches through the zona pellucida, and is thought to be a proxy for the health of the embryo [111]. The Grade 1 blastocyst has a blastocoel cavity that is less than half of the volume of the embryo; Grade 2 blastocysts have a blastocoel cavity at least half of the embryo's volume; Grade 3 blastocysts have a blastocoel cavity completely filling the embryo. Grade 4 blastocysts have a thinning zona pellucida as the blastocoel is now larger than the original embryo, although there is no breach or hatching point in the zona yet; Grade 5 blastocysts are those in which the blastocoel cavity is greater than the original volume of the embryo and the trophectoderm has started herniating through a breach in the zona; Grade 6 blastocysts are fully hatched and the embryo is outside the now empty zona pellucida [114].

The degree of hatching and the day of hatching are the main focus points of hatching assessments. In non-preimplantation genetic screening cycles with a fresh embryo transfer, an earlier day of hatching (5 vs. 6) and greater degree of hatching have shown positive associations with implantation [114]. This may be explained by more embryos hatching on day 5 being euploid than those hatching on day 6 [115, 116] or those blastocysts hatching on day 5 having greater synchrony with the endometrium than those hatching on day 6. In studies analyzing frozen embryo transfer cycles without preimplantation genetic screening, no difference in implantation rates were observed when comparing day 5 vs. day 6 hatching blastocysts [117, 118] although other studies have still found an advantage of day 5 blastocysts even when transferred in a subsequent frozen embryo transfer [119]. In addition to the size and timing of blastocoel hatching having prognostic value, the presence of string-like projections across the blastocoel which do not break down over time are associated with poorer outcomes [102].

It is important to note that the use of assisted hatching, commonly performed before the cleavage stage, significantly affects the degree of blastocyst expansion and may make the assessment of expansion less useful as embryos with less cell and/or smaller blastocoel cavities may start to herniate through the artificially hatched zona.

The inner cell mass becomes clearer as the blastocoel expands. These cells which will become the fetus and extra-embryonic structures can have morphology ranging from very large tightly packed cells to loosely aggregated smaller cells. Both Gardner and the Istanbul groups postulate that the ideal ICM, denoted by a score of A or 1, is prominent and easily discernable, containing many cells that are tightly packed together. The middle ICM category, B or 2, is composed of several loosely grouped cells. The worst category, C or 3, describes an ICM that contains very few cells that are loosely bound [111, 112, 114]. In addition, an oval ICM rather than rounded or elongated shape and an ICM larger than 4500 um^2 have been associated with higher implantation rates [120].

The trophectoderm represents the cells in direct contact with the endometrium responsible for apposition, adhesion,

and invasion of the endometrium and also eventually becomes the extra-amniotic membranes and placenta [111]. Similar to the ICM, the Gardner and Istanbul systems both evaluate the TE by the number of cells and relative cohesiveness with each other. The ideal TE, category A or 1, contains many cells that form a cohesive epithelium. The middle TE category, B or 2, is composed of few cells forming a loose epithelium. The worst category, C or 3, contains few, large cells that do not form a cohesive epithelium [111, 112, 114]. Some studies without preimplantation genetic screening have found higher implantation rates among blastocysts with better TE scores [121], although this has not been observed in others [120]. It seems rational that the quality of the cells that adhere and invade the endometrium would correlate with implantation rates.

Practically, no single component of either blastocyst scoring system is used in isolation. Rather, a composite score is created, which has been demonstrated in non-preimplantation genetic screening cycles to correlate with implantation in both fresh and frozen embryo transfers [122, 123]. However, with the increasing utilization of preimplantation genetic screening and freeze-all strategies, embryos can be given additional time for expansion before biopsy and then transferred in a subsequent frozen embryo transfer cycle with better synchrony with the endometrium. The investigation of clinically useful morphological assessments will need to be revisited in this context, which also often employs artificial hatching, to determine which morphological assessments are still clinically useful in this clinical scenario.

40.11 Conclusion

Morphological assessment of embryos for culture decision making and embryo selection is one of the most challenging aspects of IVF. To create a system that uses a series of static assessments of an extremely variable and dynamic process to create an accurate, noninvasive, reproducible, and quick grading system is a tall order. The advent of time-lapse imaging to incorporate morphokinetic analyses and capture important morphological changes and the pathways to particular checkpoints is a step in the right direction to capture key attributes of embryo development. Initial studies investigating the predictive value of morphokinetic assessments have been mixed [42, 124–127]; however, the optimal way of utilizing time-lapse imaging in conjunction with morphological [128] and molecular assessments may be ahead of us and need further elucidation with ongoing research. Various scoring systems that include different combinations of multiday evaluations have been proposed, and many have been found to be associated with improved developmental competency of the embryo [129]. However, consensus on most aspects of sequential analysis remains elusive, including the parameters used in the different scoring systems, optimal times for evaluation, predictive accuracy of the various scores, and interobserver consistency of evaluation.

Many of the timing parameters observed to favorably or unfavorably correlate with implantation will need to be revisited again in frozen embryo transfer studies to adequately address the potential confounder of embryo-endometrium dyssynchrony, which may have been encountered in studies utilizing all fresh embryo transfers. Many of the checkpoint parameters with prognostic value were identified before cryopreservation was performed as reliably as today. In these studies, many embryos reaching developmental checkpoints relatively slower were deemed less viable; however, they may be just as viable as embryos who reach certain milestones on time when placed back in sync with the endometrial environment via a frozen embryo transfer.

Just as many aspects of embryo culture must be tailored toward any specific clinic's lab, the morphological assessments employed must also be personalized to particular IVF lab's workflow, staffing, patient population, and procedures performed, as well as its culture system. Morphological assessments can improve patients and providers' expectations, increase the number of patients reaching embryo transfer, and help choose the embryo most likely to become a successful pregnancy. When done effectively, this can strengthen the argument for single embryo transfer without compromising pregnancy rates, reduce patients' time to pregnancy, and possibly also reduce treatment expenses. While new technologies with applications in gamete and embryo assessment are being developed at a rapid pace, the use of static morphological assessments will continue to be the gold standard comparator for embryo selection to which any new technique's superior utility in clinical practice will need to be demonstrated before adoption.

Review Questions

1. How many hours after conventional insemination or intracytoplasmic sperm injection should the fertilization check be performed?
   10–12
   14–16
   16–18
   18–20

2. Which are the attributes of pronuclei assessed in most pronuclear scoring systems?
   (A) Size
   (B) Symmetry
   (C) Location
   (D) Position
   (E) B, C, and D

3. True or **False**: Embryos undergoing cleavage from 1 to ≥3 cells have significantly higher implantation rates than other embryos.

4. What number of cells on day 3 of development correlates with higher rates of implantation?
   (A) 4
   (B) 4–6
   (C) 7–9
   (D) 10–12

5. Which micromanipulation technique may confound blastocyst grading?
   (A) Intracytoplasmic sperm injection
   (B) Laser-assisted hatching
   (C) Trophectoderm biopsy
   (D) Delayed stripping of cumulus cells

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Predicting Embryo Implantation Potential Using Video Monitoring by the EmbryoScope™ Time-Lapse System

Lucía Alegre Ferri, Carmela Albert Rodríguez, Sonia Pérez Albalá, and Marcos Meseguer Escrivá

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Learning Objectives

- Evolution of time-lapse due to EmbryoScope™ technology.
- Integrating EmbryoScope™ device in IVF Laboratory routine.
- The most principal time-lapse algorithms.
- EmbryoScope™ Plus. Advantages and differences.
- The contribution of EmbryoScope™ to improve the implantation rate.

41.1 Introduction

Standard methods of embryo assessment in an IVF laboratory are based on subjective morphology evaluations. These evaluations are done at discrete time points because of the negative effects that manipulation and interrupted incubation have on embryo development, but information for selecting the best embryos is limited. The efforts aimed at improving pregnancy rates have focused on the search for additional markers of viability and new methods of retrieving more information for embryo evaluation to supplement current criteria for embryo selection.

Time-lapse technology represents a powerful tool in assisted reproduction techniques. It is based on specially designed instruments which take images of the embryos at set time intervals during incubation for minimized manipulation. This technique provides additional information to the embryologist by enabling evaluation of embryos from a dynamic point of view to study exact timings of important events during embryo development.

The aim of time-lapse technology is to improve IVF success rates by having extra information to describe embryos with higher developmental potential. The embryologist therefore has a wider range of variables to rely on for the selection of viable embryos, which – all other things being equal – should result in a better embryo selection.

41.2 EmbryoScope™ Time-Lapse Technology

The EmbryoScope™ (ES) time-lapse system (Unisense FertiliTech A/S, Aarhus, Denmark) is a tri-gas oocyte/embryo incubator with a built-in microscope and camera to automatically acquire images of up to 72 individual embryos from 6 patients during development (Fig. 41.1).

Culture conditions can be set as for conventional incubators. Temperature and gas concentrations are registered and logged by an embedded PC, which automatically generates a graph for easy monitoring of the incubator conditions over time.

EmbryoScope® time-lapse instruments are designed to optimize culture environment. The system has a non-humidified incubation chamber with a very short recovery time of O_2 and CO_2 concentrations, a direct heat transfer system for stable culture temperature even at door openings, and active carbon and HEPA filters to remove VOCs and other particles. Furthermore, all system components potentially in contact with embryos or embryo environment are toxicity tested prior to release from the manufacturer.

Fig. 41.1 The EmbryoScope™ time-lapse system

41.2.1 The Microscope Imaging System

The imaging system in the ES uses low-intensity red light (635 nm) from a single LED with short illumination times for image acquisition to minimize embryo exposure to light and to avoid damaging short-wavelength light [1, 2].

Optics employed to perform the imaging of oocytes/embryos closely resemble those of an inverted microscope with Hoffman modulation contrast. However, the microscope unit is fully automated and the images are recorded as in a digital microscope. The design is much more compact than in a conventional laboratory microscope. The 20× objective lens is custom-built (Leica) and optimized at relevant wavelengths.

Digital images are recorded by a highly sensitive CCD camera (1280 × 1024 pixels) with a resolution of 3 pixels per µm, and image stacks are acquired at equidistant focal planes during embryo culture. The number of planes acquired can be selected by the embryologist (up to 17 planes).

Although recording several thousand images during embryo culture, the total exposure time in the EmbryoScope® time-lapse system during culture and acquisition of images is significantly lower than the microscope light exposure time reported for a standard IVF treatment [1].

41.2.2 Description of EmbryoSlide® Culture Dishes

The EmbryoSlide® (EmbryoSlide(R), Unisense FertiliTech, Aarhus, Denmark) holds up to 12 embryos, each in individual wells with a design patented one-to-one identification system for easy identification under the microscope

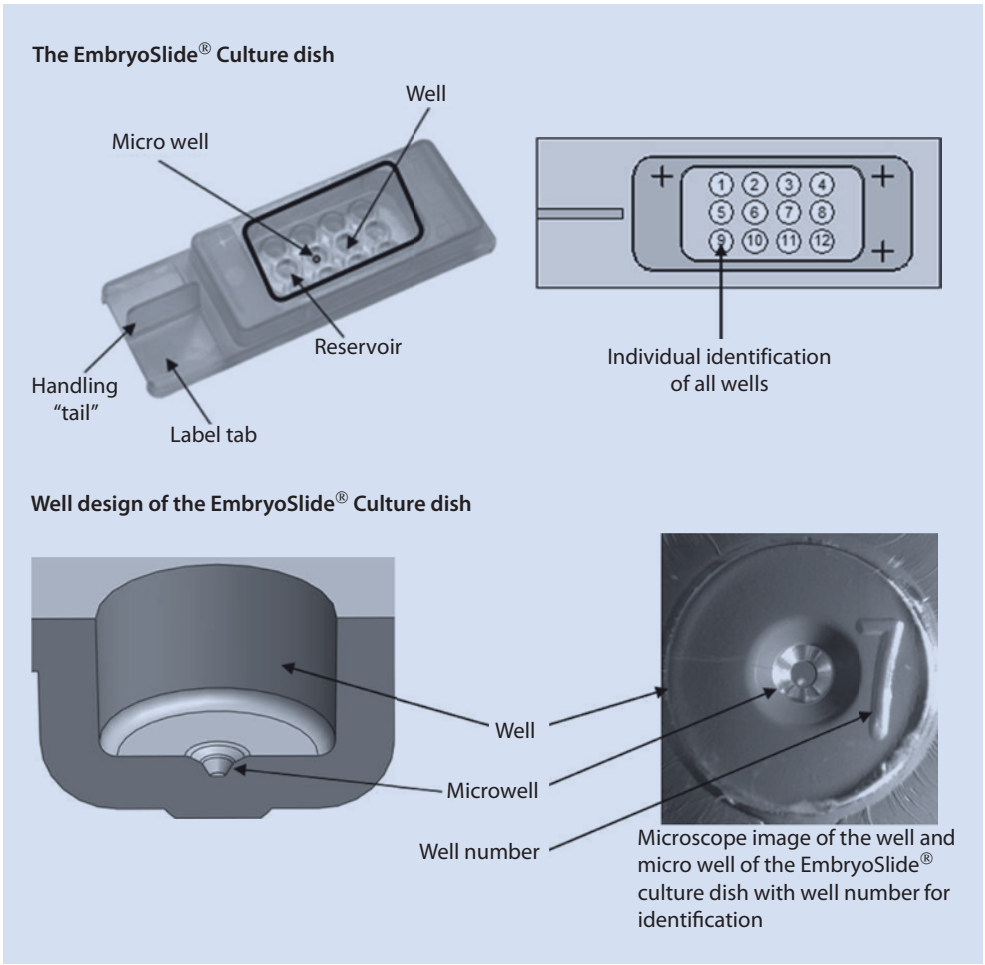


Fig. 41.2 The EmbryoSlide® design

(Fig. 41.2). The well numbers are equivalent to the embryo identifier associated to time-lapse recordings in the system. Embryos settle in a central depression at the bottom of each well (microwell) and are normally cultured in 25 µL droplets, each under a common oil overlay.

EmbryoSlide® culture dishes are packed in sterile pouches and are recommended to be prepared in a sterile, non-heated environment.

Upon media and oil loading and appropriate temperature and gas equilibration, EmbryoSlide® culture dishes can be loaded with embryos before transfer to the EmbryoScope® time-lapse system for culture and monitoring.

41.2.3 Working Procedure/Methodology

The text below describes the working procedures as performed at IVI clinics:

Oocytes are placed in the microwell just after microinjection (day 0). Therefore, the exact timing for each embryo division is calculated in hours after microinjection.

For placing embryos safely in the microwell, we use Stripper® pipettes (MXL3-275 de MidAtlantic Diagnostics, Inc.) and ensure embryo settlement at well bottom by microscope inspection.

When culturing embryos in the EmbryoScope® time-lapse system, EmbryoSlide® culture dishes containing the individually cultured embryos must be loaded in the EmbryoScope® time-lapse system both physically and electronically. Physical load is easily done by inserting into the specially designed dish holder of the embryo chamber. Electronic load is equally easily achieved at the touch of a single button on the EmbryoScope® Home screen ("Add Slide," Fig. 41.3).

Introduction of an EmbryoSlide® in the EmbryoScope® time-lapse system prompts information to be added about the patient (patient identification, date and time of fertilization) and a check for association with time-lapse images (Fig. 41.4).

It is very important to introduce the correct time at which the microinjection has been performed, as all recorded events will be defined taking that time as starting point.

In the table that appears in the right-hand side (Fig. 41.4), we add any information related to each particular embryo.

Once the EmbryoSlide® culture dish has been placed inside the embryo chamber, the software automatically

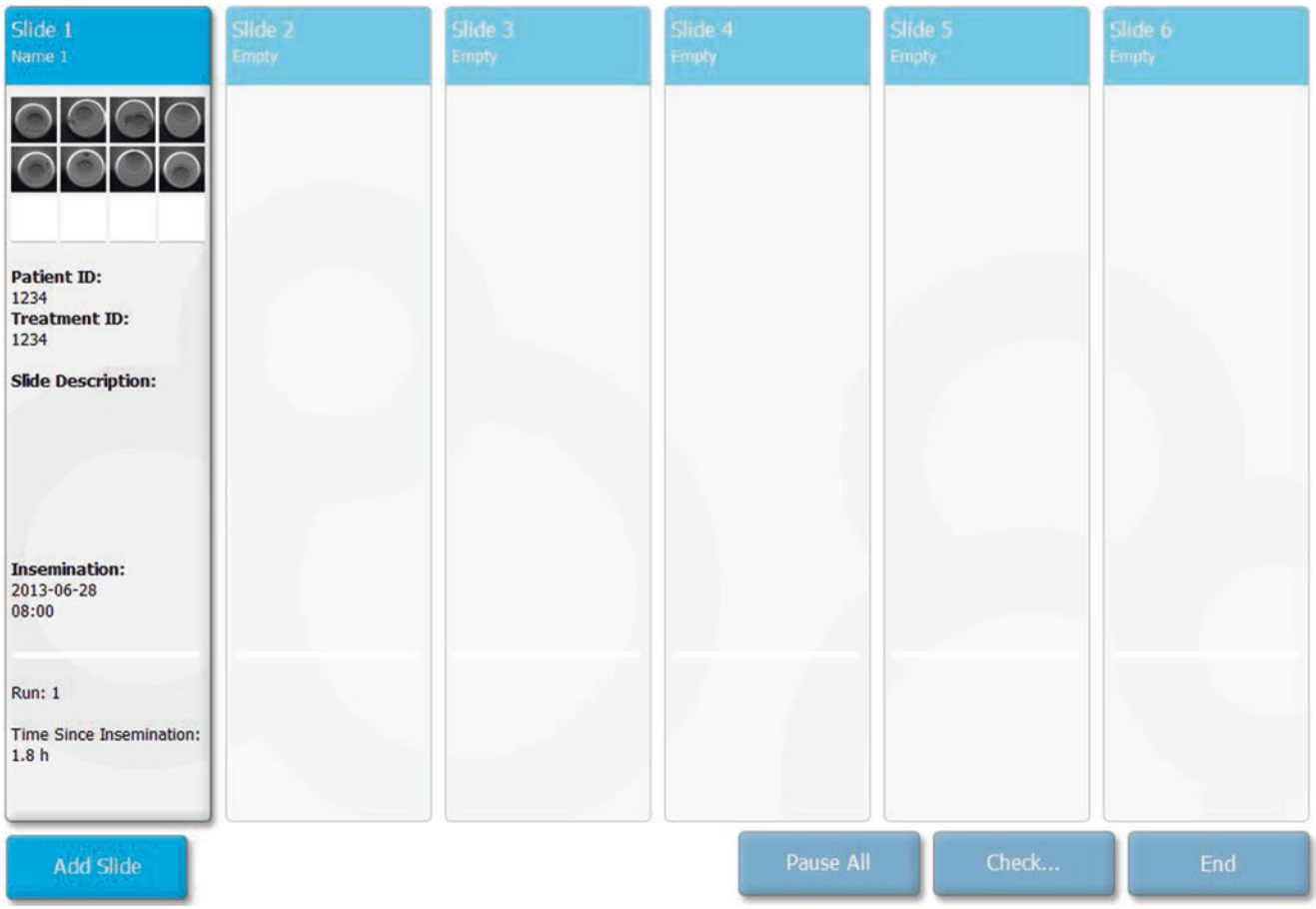
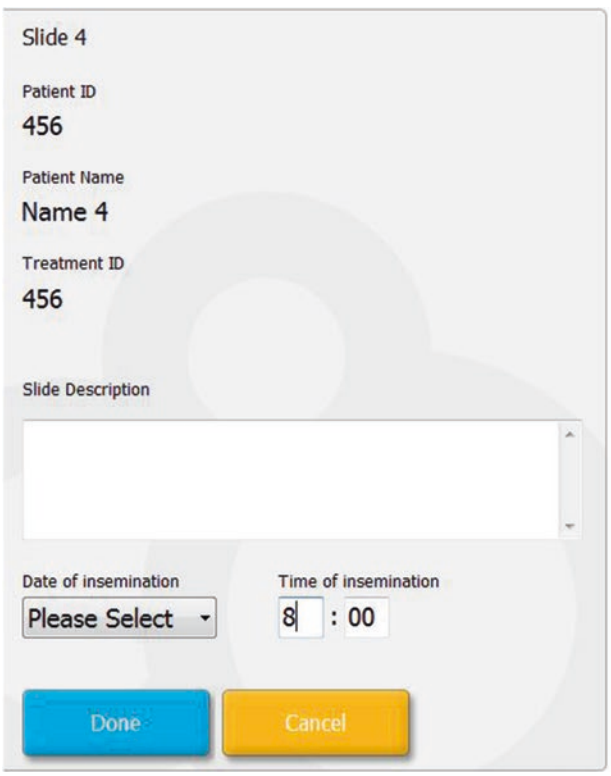


Fig. 41.3 EmbryoScope® Home screen



Well	Camera	Embryo ID	Description
1	OK	1	
2	OK	2	
3	OK	3	
4	OK	4	
5	OK	5	
6	OK	6	
7	OK	7	
8	OK	8	
9	OK	9	
10	OK	10	
11	OK	11	
12	OK	12	

Fig. 41.4 EmbryoScope™ screen with patient identification, date and time of fertilization

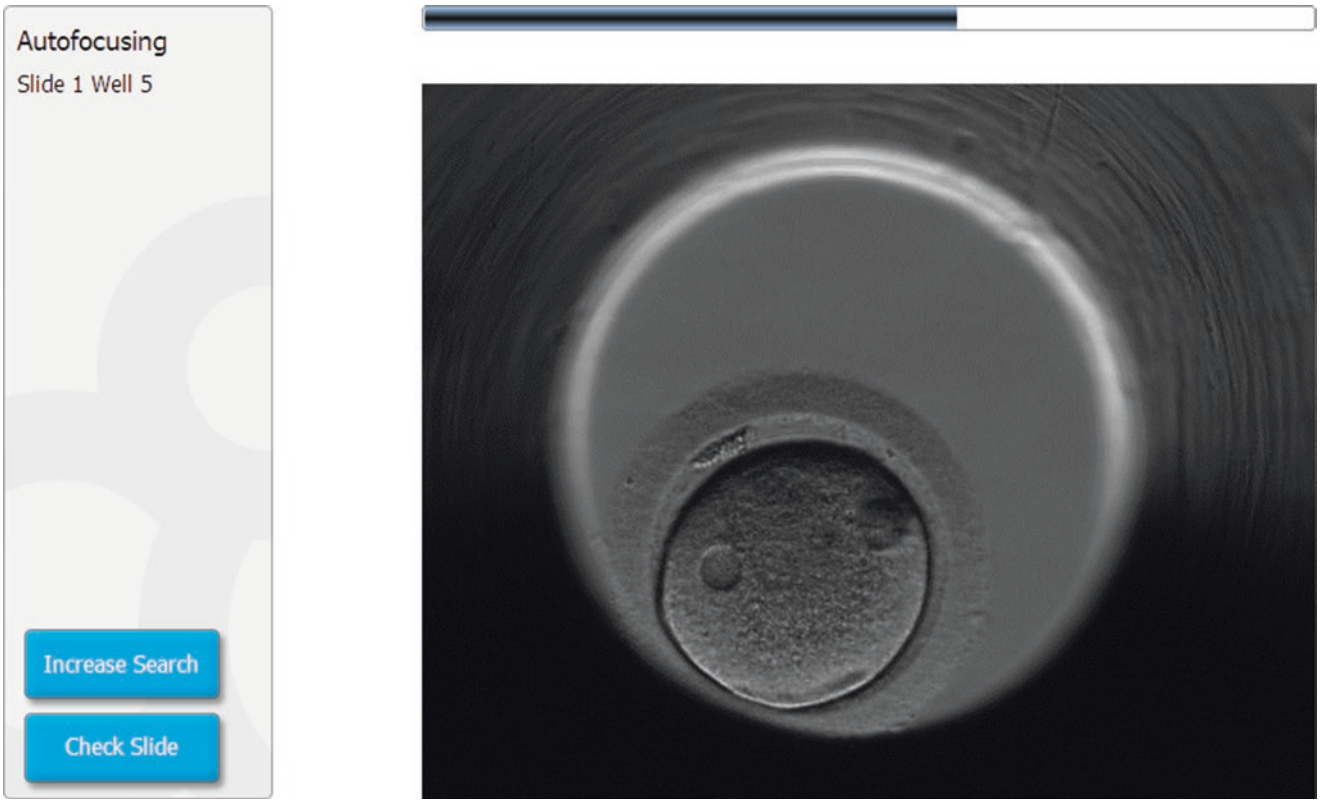


Fig. 41.5 Autofocusing wells system

locates the wells of the dish and most informative focal plane (Fig. 41.5). After autofocusing, the system starts time-lapse image acquisition from each well in multiple focal planes as defined by the user. We sometimes use the refocus option ("New Focus") provided by the system to manually settle the focus plane (Fig. 41.6).

While the instrument is running, an overview of the cultured dishes is presented on the Home screen with the option to access details about culture conditions and each EmbryoSlide® culture dish (Fig. 41.7).

By pressing a Slide tab, we can view the full slide with the last image recorded in each well. Such an overview of the embryo development of a particular patient is very informative and useful for a quick overview of instrument content. It is also possible to focus on an individual embryo by a single touch on the screen which retrieves last image and more detailed information of that particular embryo. This feature includes several options including a live view, a video playback function which will play the entire time-lapse video of the particular embryo, and navigation within the current EmbryoSlide® culture dish (Fig. 41.8).

All data related to each EmbryoSlide® culture dish, including time-lapse images and incubation summaries, are saved in an integrated PC and on the appertaining EmbryoViewer™ workstation (EV).

41.2.4 Image Analysis Using the EmbryoViewer™ Software

The EmbryoViewer™ software (Unisense FertiliTech A/S, Aarhus, Denmark) is an advanced software specially designed for the EmbryoScope® time-lapse system for image analysis of the time-lapse acquisitions recorded by the system. Using this software, all embryo developmental events throughout the entire culture time (up to blastocyst stage) are annotated together with the corresponding timing of the events in hours after microinjection (Fig. 41.9). Blastomere divisions are routinely marked by the embryologist as well as events characteristic of fertilization, compaction and blastulation, multinucleation, unevenness in blastomere size, and degree of fragmentation.

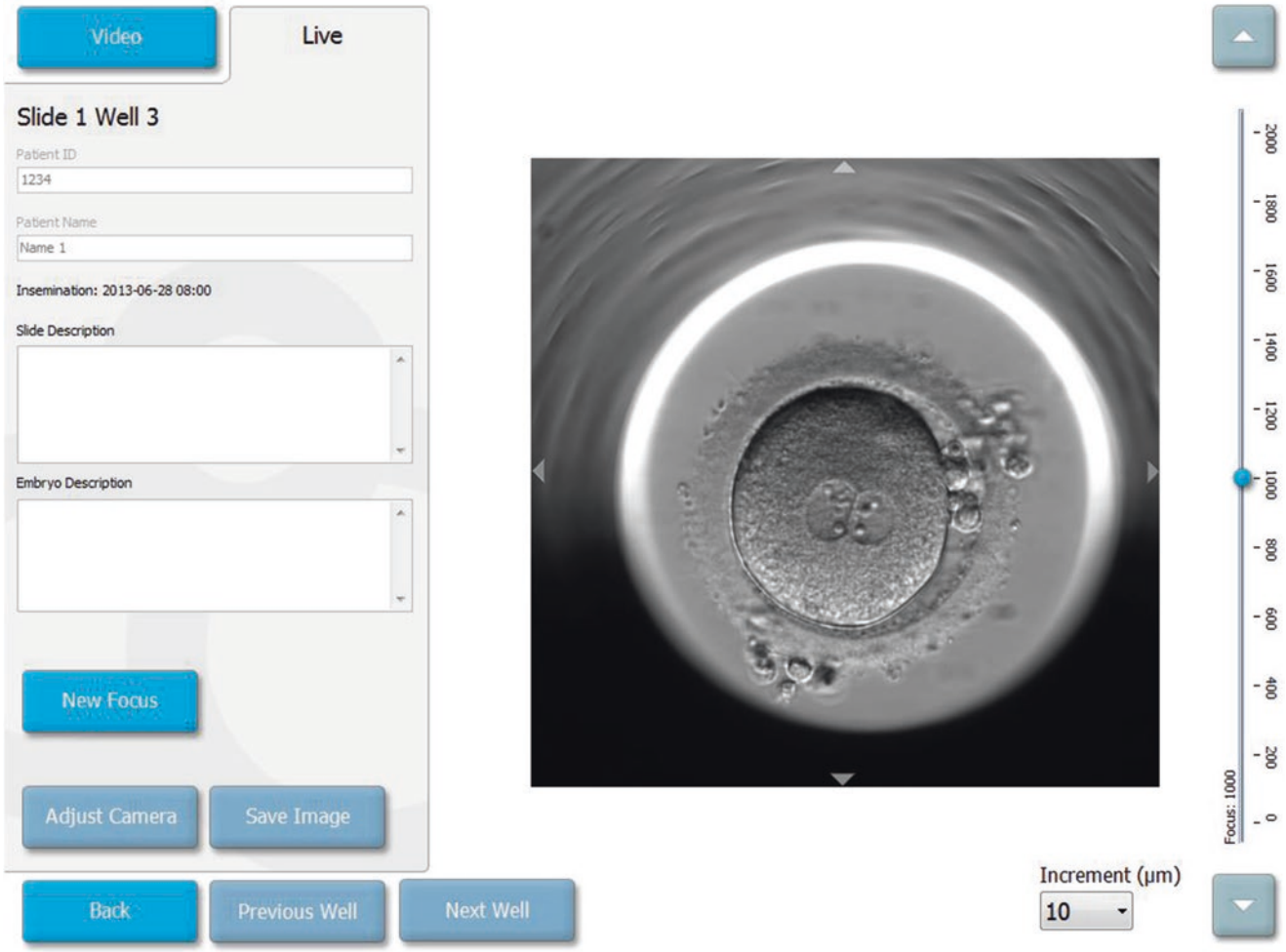


Fig. 41.6 Manual system for new embryo focus

At all our centers, we apply the embryo evaluation algorithm designed by Meseguer et al. [3]. Using the Compare & Select feature of the EmbryoViewer™ software, which ranks embryos according to user-defined criteria, this automatically provides the quality grade or category for each embryo according to the algorithm (Fig. 41.10).

The EmbryoViewer™ workstation provides the user with decision tools to mark embryo images once the selection of which embryos to be used for transfer and freezing or kept in the laboratory for further observation is performed (green for transfer, blue for freezing, red for not viable, and yellow for observation) (Fig. 41.11).

The system also provides the option to generate report formats of the embryos selected for transfer and freezing which can be tailored to clinic design and printed. We use the "Patient Report" for presenting the patient to images of the development of the embryos chosen for transfer and the final image acquired of embryos that are going to be cryopreserved. Moreover, a video of the complete development can be generated from the recorded stack of images and provided to the patients.

41.3 EmbryoScope™ Plus: New Version

EmbryoScope™ is the world's most used time-lapse system for IVF since 2009.

This year Vitrolife® has launched their new time-lapse system incubator: EmbryoScope™ Plus (Fig. 41.12). This new device has been validated and used clinically in five Scandinavian clinics, after having been involved in more than 500 treatments.

The main advantages of EmbryoScope™ Plus:

- Large capacity.
- Small footprint.
- Efficient workflow.
- Resource optimization.

EmbryoScope™ Plus has a capacity for 15 patient culture dishes and each of them can get up to 16 embryos. This would represent more than double the patient capacity, compared to other bench-top time-lapse systems and three more than previous EmbryoScope™ version. EmbryoScope™ Plus is

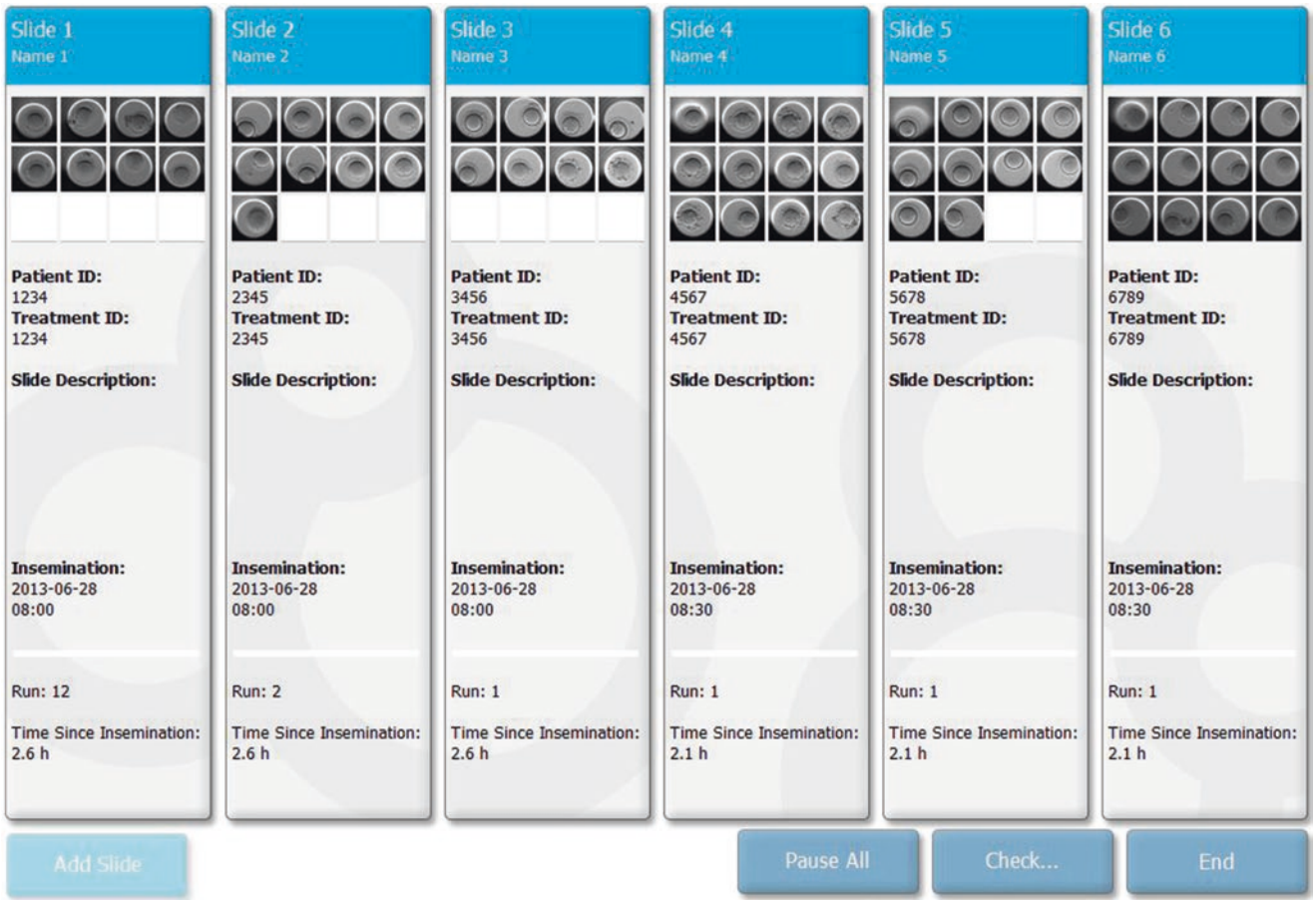


Fig. 41.7 Home screen with details about culture conditions of each EmbryoSlide® culture dish

used with an EmbryoViewer™ Software version allowing you to see all 16 embryos at a time.

To improve workflow, the EmbryoSlide® Plus culture dishes are automatically registered using a special patient barcode labeling system (Fig. 41.13). A patient identification barcode system offers a flexible and efficient workflow. The device automatically reads and registers entry of a new patient and reentry of an existing patient slide.

41.4 Overview of Studies Undertaken to Date Using EmbryoScope™

The study presented by Cruz et al. was the first step to evaluate whether the EmbryoScope™ time-lapse system provides a safe and controlled environment compared with conventional incubators [4]. In this study, embryo selection for transfer was based on morphological evaluation at the same time points for both the time-lapse system and the conventional incubator groups, the additional information provided by the time-lapse system not being taken into account.

Results obtained showed no significant variation between the two incubator groups when comparing embryo quality, development, and ongoing pregnancy rates, which confirmed the safety of the system for clinical use.

Kirkegaard et al. [5] also developed a randomized clinical trial to evaluate the safety of the EmbryoScope™ time-lapse system before introducing the instrument in a clinical setting. Results obtained in this study confirm no differences on development, clinical pregnancy, or implantation rates between incubation in the EmbryoScope™ system and a conventional incubator. With these results, they concluded that EmbryoScope™ time-lapse system supports embryonic development equally and can therefore be introduced clinically providing a potential tool for embryo evaluation [5].

Upon demonstration of the safety as an incubator with controlled incubation conditions, the next purpose of Meseguer's group was to generate and evaluate a tool for the selection of viable embryos based on the exact timing of embryo development events together with morphological patterns by using EmbryoScope™ time-lapse system to monitor embryo development. They presented the largest data set on transferred ICSI-generated embryos analyzed by time-lapse, to correlate morphokinetic parameters with implantation and pregnancy rates [3]. The precise timings of numerous morphokinetic parameters were identified where

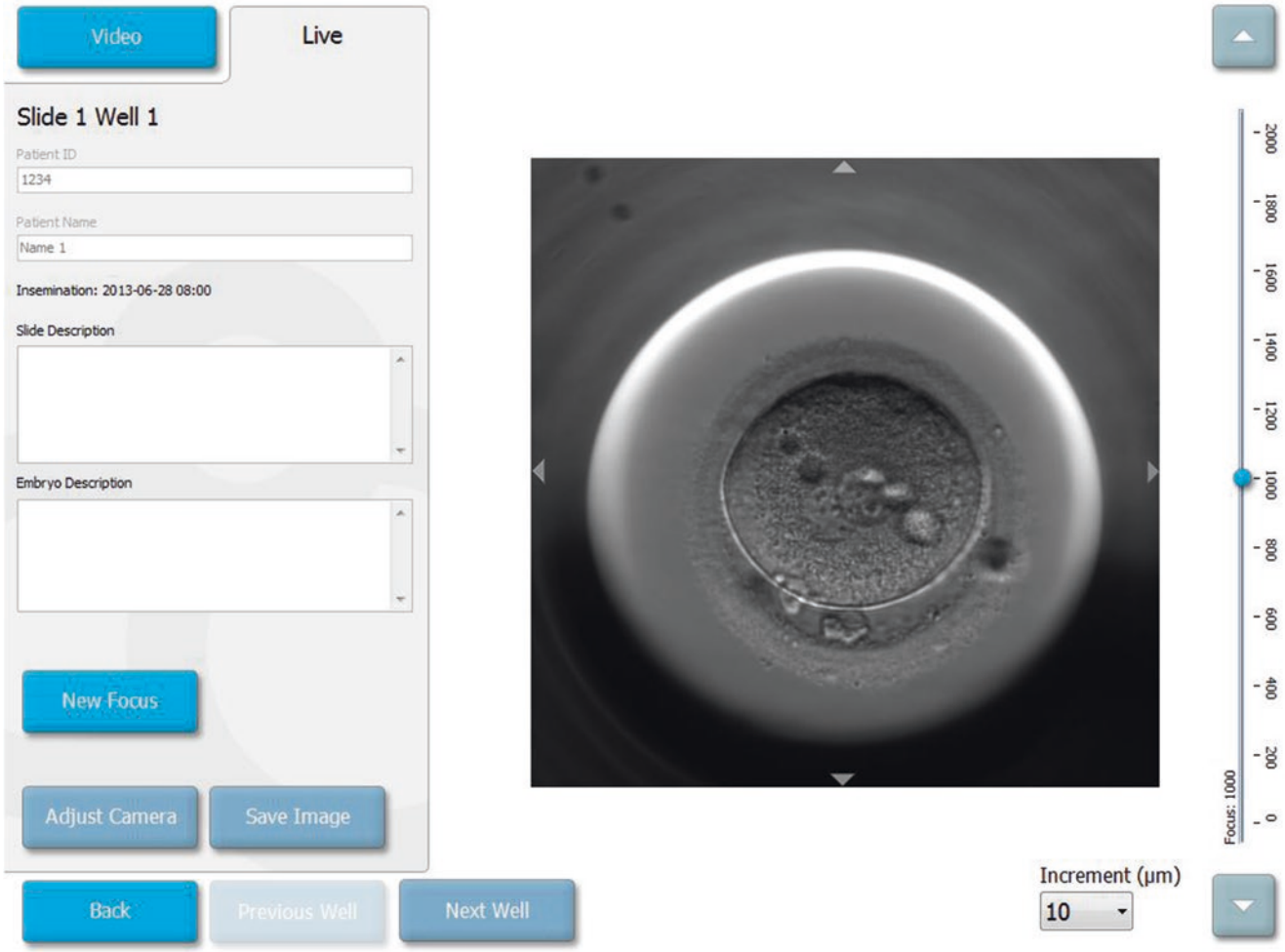


Fig. 41.8 Time-lapse video and navigation within the current EmbryoSlide® culture dish

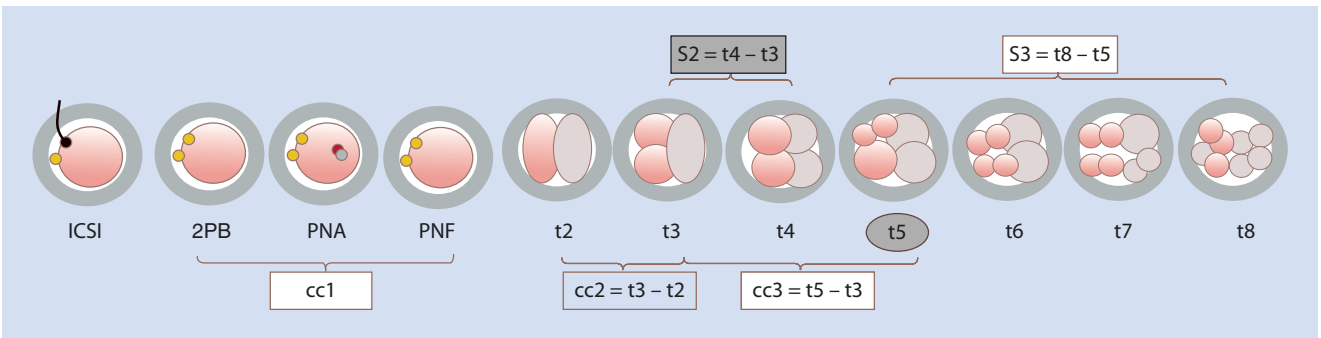


Fig. 41.9 Morphokinetic parameters of embryo development

time zero was the time of microinjection. Division into two cells was defined as t2, into three cells t3, into four cells t4, and into five cells t5, defining time of cleavage as the first observed time point when the newly formed blastomeres were completely separated by clearly defined membrane. Cell cycle duration was also evaluated, the second cell cycle (cc2) being the duration of the period as a two-blastomere embryo (i.e., t3 − t2) and the second synchrony (s2), synchrony in division from two-blastomere embryo to four-blastomere embryo (i.e., t4 − t3). The four quartiles for the timing of each of these parameters were compared with the percentage of successfully implanting embryos in each of the quartiles. Optimal ranges were established considering the two consecutive quartiles with the highest implantation probabilities in each category. Statistical analysis defined the most relevant parameters for embryo evaluation. The results obtained were

Fig. 41.10 Quality grade or category for each embryo according to the algorithm defined

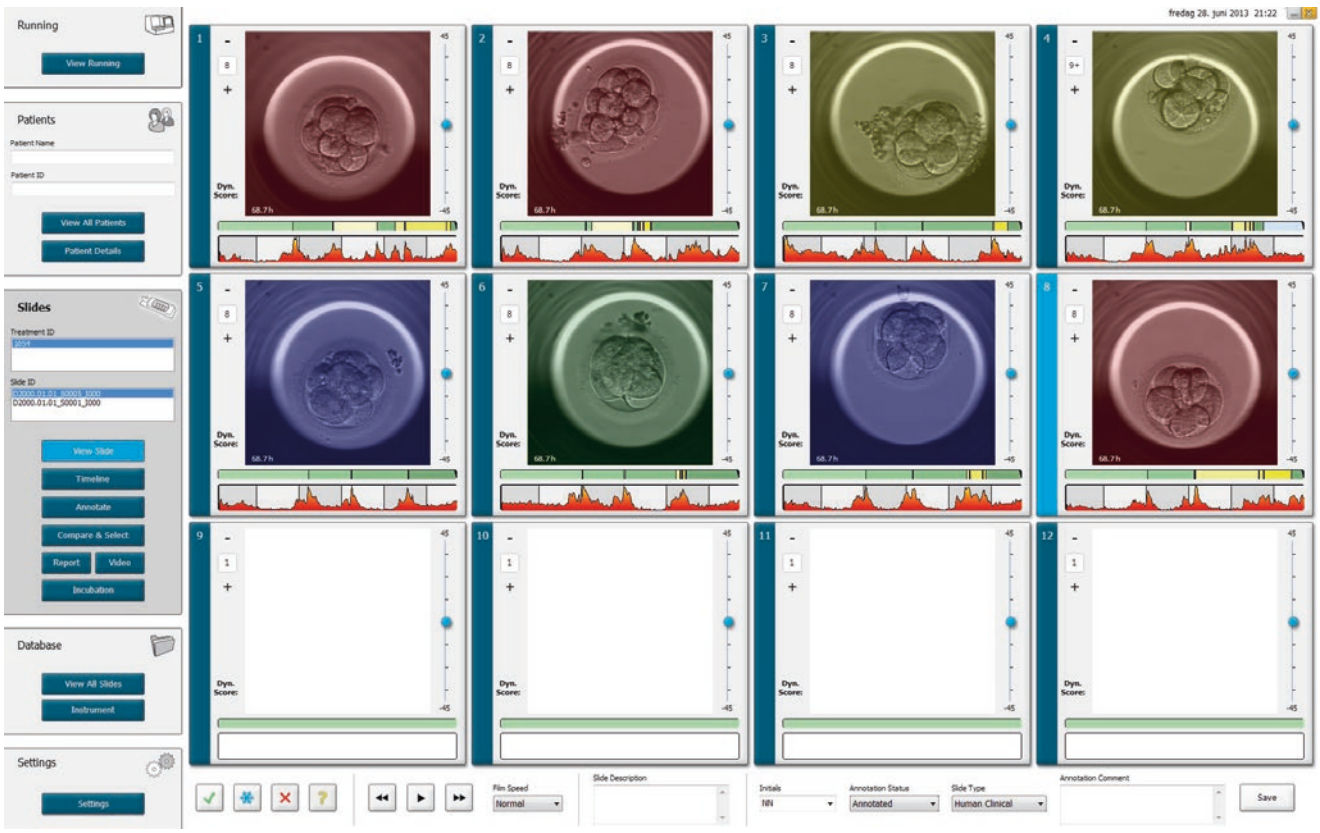


Fig. 41.11 Decision tools to mark embryo images: green for transfer, blue for freezing, red for not viable, and yellow for observation

Fig. 41.12 Vitrolife® new time-lapse system incubator: EmbryoScope™ Plus

Fig. 41.13 Special patient barcode labeling system: EmbryoScope™ Plus

then used to design a hierarchical classification algorithm for the selection of embryos with the highest implantation potential. The timing of the cleavage to five cells turned out to have the best correlation with implantation success and was superior to the timing of the first cell division used in earlier studies of early cleavage [3].

The algorithm starts with a morphological screening of the embryos to discard the embryos that are clearly not viable and which are consequently classified as category F. Another point to be considered in implementing this algorithm is to exclude embryos fulfilling at least one of the following exclusion criteria: (i) uneven blastomere size after the first cleavage (when the embryo has two cells) (blastomeres were considered uneven if the average diameter of the large blastomere was 25% larger than the average diameter of the small blastomere), (ii) abrupt division from one to three cells, or (iii) multinucleation at the four-cell stage (category E). After evaluation for these criteria, the remaining levels of the hierarchy are defined according to the defined ranges of some of the variables (t5, s2). Once the embryo is classified with a specific grade, the timing of cc2 is taken into account, adding a plus (+) if it is inside the range established as optimal or minus (−) if it is outside this optimal range. In this way, embryos are graded $A^+ - D^-$, E, or F using the algorithm (Fig. 41.14).

One of the exclusion criteria defined in Meseguer's algorithm was studied in detail by Rubio et al. [6]. By using the EmbryoScope™ time-lapse system, they precisely identified each cell cycle and observed abnormal lengths of the cell cycle, such as the abrupt cleavage of human embryos from two to three cells in less than 5 h, defined as a direct cleavage (DC 2–3) (Fig. 41.15). Clinical pregnancy and implantation rates of embryos undergoing abrupt cell division from two to three cells were studied to evaluate the clinical applicability of this morphokinetic parameter. The known implantation rate of DC 2–3 embryos was statistically significantly lower than for embryos with a normal cleavage pattern. The incidence of direct cleavage was about 14% in the total embryonic cohort which, combined with the markedly decreased implantation rate of direct cleavage, suggests this to be a strong morphokinetic parameter for clinical use.

Using this algorithm and the EmbryoScope™ time-lapse system, Basile et al. compared different culture media, demonstrating identical timings and proportion of optimal embryos for the two media analyzed [7]. These results validate the algorithm for embryo selection developed by Meseguer et al. [3] in diverse culture conditions.

The further use of the described algorithm by Meseguer et al. [3] is in the largest retrospective study to date on pregnancy outcomes comparing conventional incubation with time-lapse technology [8]. Even though prospective studies are obviously preferable, this was a first approximation where all the available data from ten different IVI clinics was analyzed, adding up to a large quantity of heterogeneous

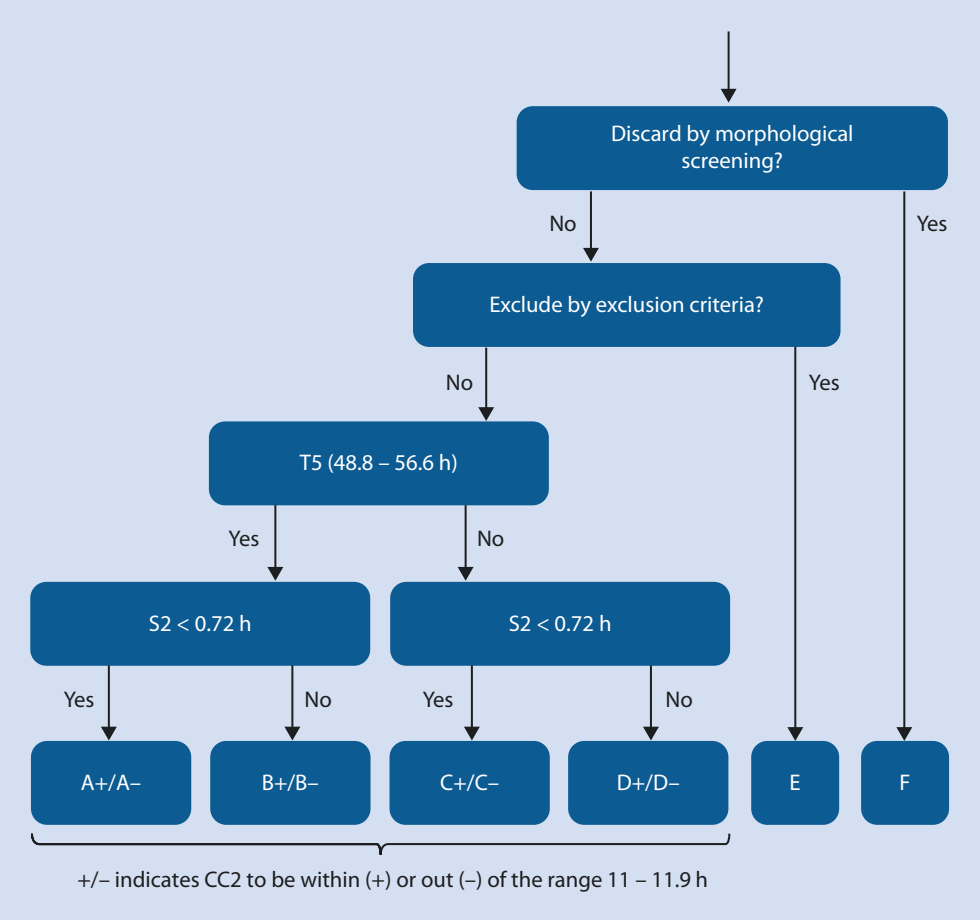


Fig. 41.14 Hierarchical classification algorithm for the selection of embryos with the highest implantation potential. Meseguer M.

In this figure are represented the levels for algorithm implementation. The morphological assessment and the exclusion criteria can classify embryos as E or F. The embryos which pass this initial screening and are not discarded will be included in eight categories +/– (A+/–, B+/–, C+/–, D+/–), according to the most relevant morphokinetic parameters in order of relevance: T5 (48.8–56.6 h), S2 (< 0.72 h) and CC2 (11–11.9 h)

information. A key limitation of this study, because of its retrospective design, was that patients were not evenly or randomly distributed between the two different incubators, which can affect and distort final conclusions. Because of this, it was essential to analyze for any confounding factors that may significantly influence pregnancy rates and their uneven distribution between incubators. Oocyte source, maternal age, day of transfer, and number of retrieved oocytes were identified as significant confounding factors and included in a logistic regression model. The results obtained suggested a relative 20% improvement in pregnancy rates in the time-lapse incubator system compared with the conventional incubator, which implies a larger impact than the improvement associated with blastocyst culture [8]. For treatments with conventional incubators, the average clinical pregnancy rate per initiated cycle with oocyte retrieval was 44.9%. If they had been incubated using time-lapse, a relative improvement of 20% would imply an expected clinical pregnancy rate for the same cycles of 53.9%, an absolute improvement of 9%.

Those results needed to be confirmed with prospective studies which have already been started. In 2014 Rubio et al. [9] presented a prospective study to determine if the combination of EmbryoScope™ time-lapse monitoring system (TMS) and morphokinetic multivariable models implies an outcome clinics improvement versus conventional incubator culture and an exclusive classic morphological analysis. Significant differences were found according to ongoing pregnancy rate 51.4% (95% CI, 46.7–56.0) vs. 41.7% (95% CI, 36.9–46.5), early pregnancy loss 16.6% (95% CI, 12.6–21.4) vs. 25.8% (95% CI, 20.6–31.9), and implantation rate 44.9% (95% CI, 41.4–48.4) vs. 37.1% (95% CI, 33.6–40.7), TMS and CI, respectively. This publication placed the EmbryoScope™ at the top of incubation systems.

Basile et al. [10] in line with the results obtained by Meseguer et al. conducted a multicenter study in which a total of 1664 oocyte donation cycles were included. Cleavage timing and cellular cycles were analyzed in all embryos. A significant correlation was established between t3, cc2, and t5 parameters and implantation rate. They also identified three events that affected the embryo quality: direct cleavage from one to three cells, uneven blastomere size in cc2, and multinucleation in cc3. According to these results, an improved algorithm version classified the embryos into five categories related to implantation potential (A, 32%; B, 28%; C, 26%; D, 20%; and E, 17%, $p < 0.001$).

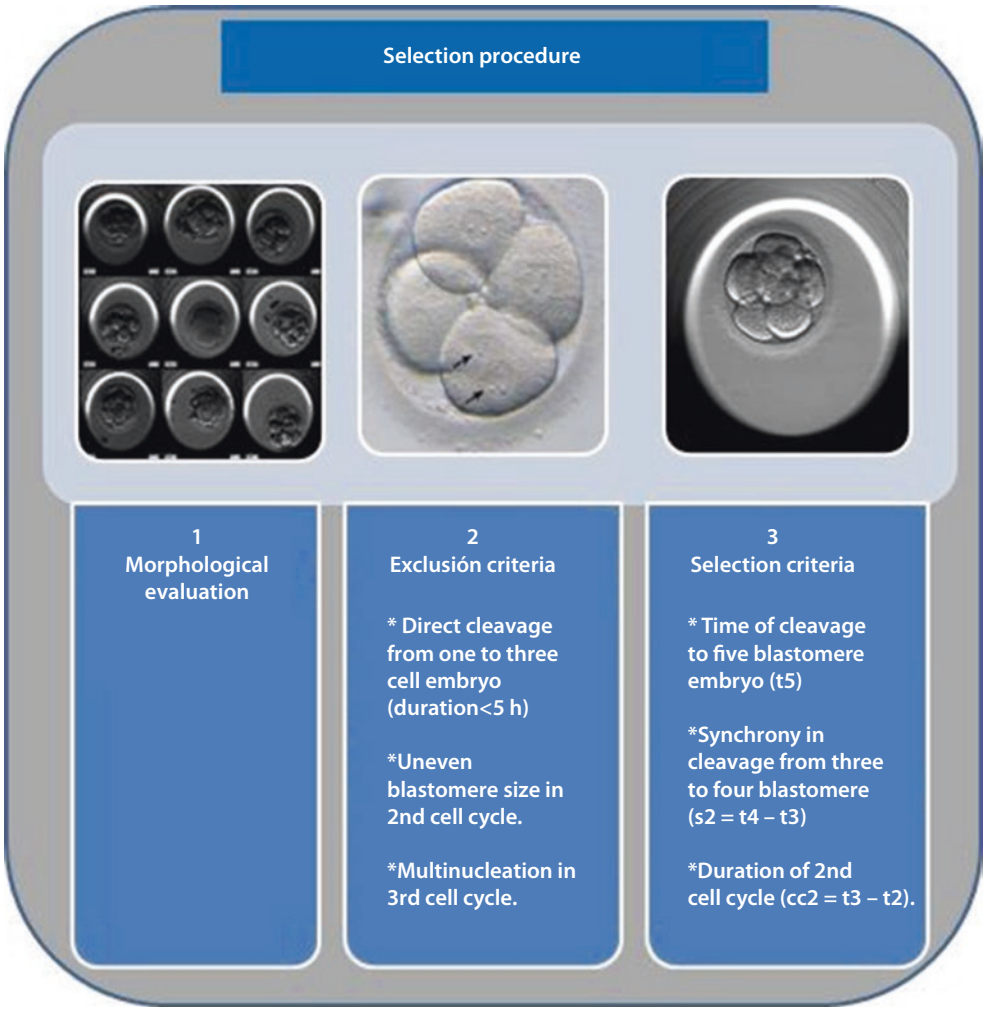


Fig. 41.15 Exclusion and selection criteria based on time-lapse markers

In order to demonstrate associations between various cleavage-stage kinetic parameters and the ability of the embryos to reach the blastocyst stage, Cruz et al. [11] compared the blastocyst rate and morphological features for cleavage-stage embryos, which were graded according to their morphokinetic development. The blastocyst rate and morphology were highest for embryos from the best morphokinetic grade A and gradually decreased to the lowest rates and qualities for the lowest grade D. From their results, Cruz et al. concluded that time-lapse-based evaluation of the exact timing of early events in embryo development is a promising tool for the prediction of blastocyst formation and quality.

Based on a thorough study of the EmbryoScope®-acquired time-lapse images, Dal Canto et al. [12] found human embryo cleavage rates to be suggestive of their ability to develop to the blastocyst stage and to implant. They showed similar cleavage patterns up to the six-cell stage of embryos that later developed into blastocyst and those that did not, but highlighted the importance of timings of cleavage to the seven- and eight-cell stages for predicting blastocyst potential. In this study implanting embryos developed to the eight-cell stage in a significantly shorter period than those unable to implant. With these results, they concluded that cleavage from the two- to the eight-cell stage occurs progressively earlier in embryos with the ability to develop to expanding blastocysts and to implant [12].

After the study of 17 parameters of embryo development in blastocysts incubated in EmbryoScope™ time-lapse, Motato et al. [13] created the largest database of human blastocysts (a total of 7.843 embryos) and two independent models. In a first study phase, they observed that if tMC and t8 − t5 took place between 81.28 and 96.0 and ≤8.78 h after ICSI, respectively, the probability of blastocyst formation was higher. Furthermore, a second model of implantation potential was published considering t8 − t5 and tEB as key morphokinetic parameters. The implantation rate was significantly higher when tEB happened between 107.9 and 112.9 h and t8 − t5 had a length ≤5.67 h after ICSI. The concept that blastocyst formation and implantation should not be ruled by the same parameters was already established in 2014 by Desai et al. [14]. The morphokinetic mechanisms registered in each event were different. Other models emerged from embryo morphokinetic study; this was the case of Milewiski et al. [15], those who identified t2, t5, and t3 − t2 as relevant markers of blastocyst formation likelihood. Thanks to EmbryoScope™,

Mizobe et al. [16] observed that when two-cell and three-cell stage took place in 25.90 and 37.88 h, the quality of blastocyst was higher and had more likely to show "B" or "A" categories in their inner mass and trophectoderm.

The multinucleation in blastomeres of embryos from two to eight cells during embryo development is the subject of interest, and it has long been studied. Aguilar et al. [17] in accordance with that established by Basile et al. identified the multinucleation as an event which can influence embryo quality. For that reason, Aguilar et al. presented a retrospective EmbryoScope™ survey with the aim of finding a possible relation among multinucleation and implantation rate. They observed that S-phase length average in cc1 of KID+ embryos was longer than in KID- cases (15.50 h vs. 14.38 h) and a little shorter in cc2 (8.35 h vs. 8.60 h), respectively. Furthermore, multinucleated embryos at four-cell stage showed a lower implantation rate; however it was not significant. So they concluded that the reason that multinucleation and implantation rate was not correlated as due to self-correction event is much more common than we might think. This theory is backed up by other research groups.

The study published by Balakier et al. [18] defined recently published a study where it was observed that multinucleation process had the same incidence in both euploid and aneuploid embryos. They analyzed a total of 1,500 embryos, which were biopsied in D5 and incubated in EmbryoScope™ incubator. After conducting an exhaustive analysis, it was established that, despite the embryos with multinucleated blastomeres experimented later division in early stages than mononucleated embryos, no differences in aneuploidy percentage was found in blastocyst stage. Only women's age showed a significant correlation with multinucleation events.

The EmbryoScope® time-lapse system has been demonstrated of great value in assisting more resource-consuming laboratory procedures due to the precise and continuous recordings of embryonic development.

Preimplantation genetic diagnosis encourages further investigations using time-lapse systems such as the impact of blastomere removal and correlation of morphokinetic parameters with aneuploidy risk.

Kirkegaard et al. presented the first study to evaluate the effect of blastomere biopsy on early human embryonic development using time-lapse analysis. Using the EmbryoScope™ time-lapse, they demonstrated that although cleavage-stage embryo biopsy prolongs the cell stage at which the biopsy is performed, the cleavage rate after this initial delay seems unaffected [19]. Biopsied and non-biopsied embryos started hatching at the same time, which caused duration of the blastocyst stage to be shorter in the biopsied group, due to a shorter time from reaching the full blastocyst stage until hatching. Moreover, the study confirms previous findings from studies on assisted hatching, showing the embryo to hatch through the artificial breach in the zona pellucida. The study hereby contributes to the understanding of the effect of blastomere biopsy and the early development of the human embryo.

Table 41.1 Classification of ploidy with associated incidence rates and probability of aneuploidy

Risk class	No of embryos	Incidence of aneuploid embryos	Probability of an embryo being aneuploid
Low $t_B < 122.9$ h and $t_{SB} < 96.2$ h	36	0.36	0.37
Medium $t_B < 122.9$ h and $t_{SB} \geq 96.2$ h	49	0.69	0.69
High $t_B \geq 122.9$ h	12	1.00	0.97

To evaluate the difference in morphokinetics between aneuploid and euploid embryos [20], Campbell et al. (2013) compared morphokinetic variables of these two groups of embryos using EmbryoScope® time-lapse images. Their results showed that multiple aneuploid embryos were delayed at the initiation of compaction (t_{sc}) and the time to reach full blastocyst stage (t_B). Embryos having single or multiple aneuploidy also presented a delay in initiation of blastulation (t_{SB}) compared with euploid embryos [20].

Morphokinetic variables found to differ between euploid and aneuploid embryos were used to build a risk classification model by classifying the embryos into groups depending on the value of the variables t_{SB} and t_B and finally developed the following algorithm: low risk, $t_B < 122.9$ h and $t_{SB} < 96.2$ h; medium risk, $t_B < 122.9$ h and $t_{SB} \geq 96.2$ h; and high risk, $t_B \geq 122.9$ h (Table 41.1).

This same group also developed a retrospective analysis to evaluate the effectiveness and potential impact of this classification model for unselected IVF patients without biopsy and preimplantation genetic screening (PGS) [21].

When the aneuploidy risk classification model was applied, the medium- and low-risk classes for aneuploidy were significantly different from each other with respect to known implantation rates (Table 41.2). Of the few embryos classified as high risk subsequent to transfer, none implanted. The aneuploidy risk classification model, therefore, indicated the predictive power of this algorithm to identify and avoid selecting embryos at high risk of implantation failure using noninvasive objective criteria.

Soon after, other researchers used time-lapse technology to identify morphokinetic parameters that could be markers of chromosomal aneuploidies. The morphokinetic differences between euploid and aneuploid embryos were also described by Chawla et al. [22] in a retrospective study. Basile et al. [23] already in 2013 created a logistic prediction model through a prospective study in PGS embryos that were incubated in EmbryoScope. On that model, t5 − t2 (>20 h), cc3 (11–18 h), and t5 (47–58 h) were proposed as health genetic

Table 41.2 Known implantation data rate for fetal heart beat and live birth for each aneuploidy risk class

Risk class	Fetal heart beat in known implantation data	Live birth in known implantation data
Low $t_B < 122.9$ h and $t_{SB} < 96.2$ h	72.7[a] ($n = 33$)	61.1[b] ($n = 18$)
Medium $t_B < 122.9$ h and $t_{SB} \geq 96.2$ h	25.5[a] ($n = 51$)	19.2[b] ($n = 26$)
High $t_B \geq 122.9$ h	0 ($n = 4$)	0 ($n = 2$)

[a] $p < 0.0001$
[b] $p < 0.01$

embryos indicators. With the same objective in mind, in 2014 the same research group analyzed 504 embryos from 77 patients that were subjected to array comparative genome hybridization (CGH) in day 3 of development. In this case, the same correlation was found between morphokinetic timing events and genetic endowment of the embryos: t5 − t2 OR = 2.853 (95% CI 1.763–4.616), followed by cc3 OR = 2.095 (95% CI 1.356–3.238). According to these findings, they created an algorithm which classified the embryos into four categories (A to D) by t5 − t2 and cc3 development parameters. Each category exhibited significant differences in the percentage of euploidy embryos (A, 35.9%; B, 26.4%; C, 12.1%; D, 9.8%).

Recently, other researches as Patel et al. [24], according to Basile algorithm, found differences between morphokinetic parameters of euploid and aneuploid embryos (t5, 28.6% vs. 17.5%; cc3, 25.9% vs. 20.8%; and t5 − t2, 26.7% vs. 14.3%). Excellent results were also registered by Yang et al. [25] in trophectoderm biopsied of day 5 blastocysts. They formed two groups: time-lapse and conventional incubator group. Time-lapse parameters were used as powerful tool in embryo transfer selection, since implantation rate, clinical pregnancy rate, and ongoing pregnancy in these patients were significantly higher (66.2% vs. 42.4%, $p = 0.011$; 71.1% vs. 45.9%, $p = 0.037$; and 68.9% vs. 40.5%, $p = 0.019$) in time-lapse and conventional incubator group, respectively. The time-lapse technology and PGS combination is a great strategy to improve clinical results.

Other field, where time-lapse opened newest investigation lines, is collapse events taking place in blastocyst stage. Monitoring images allow us to detect and quantify collapse events during embryo development.

Brodi et al. [26] performed a study to clarify if blastocyst collapse episodes are in some ways correlated with live birth rate (LBR). Three collapse blastocyst groups were defined: no, single, or multiple. They observed that LBR was lower as collapse events were more frequent: 36% (no collapse group), 31% (single), and 14% (multiple). However, that collapse events are not predictive markers was determined.

Most recently, at the 32nd Annual ESHRE congress, Galán et al. [27] presented a re-expansion blastocysts survey. A total of 435 thawed-warmed KID blastocysts were analyzed. Embryoscope incubator was used to study re-expansion blastocyst after warming until transfer. The variables included were ZP thickness (μm), blastocyst area (μm^2), inner cell mass (μm^2), and collapse events. Significant differences in implantation rate were observed depending on ZP thickness and blastocyst area. These results showed that time-lapse analysis offers new implantation markers for warmed embryos.

One of the great advantages by time-lapse technology is the option to study all embryo development retrospectively. Domínguez et al. [28] wanted to check if it was a relationship between interleukin (IL) 6 and the duration of cellular cycles. To that end, they compared morphokinetic parameters with presence/absence of IL-6. A correlation was found between the duration of cc2 and IL-6 presence. These two parameters were combined to generate a hierarchic model including four categories: A, B, C, and D. They observed that embryos with the presence of IL-6 and 5–12 h cc2 duration showed a significant higher implantation rate.

In summary, time-lapse systems improve our knowledge of the real time distribution of the main events of embryo development making it possible to establish, with accuracy and objectivity, the basis of the embryo development timing. The EmbryoScope® technology provides flexibility in working routines and evaluation protocols due to the platform design. This technology might also improve effectiveness of IVF cycles increasing the ability to identify embryos with higher implantation potential, in order to perform elective single embryo transfer reducing multiple pregnancy rates.

Review Questions

1. Which is the main reason that you should consider if you have (or plan to have) an EmbryoScope™ incubator in your IVF laboratory?
   (a) To improve the embryo selection provided by morphology.
   (b) To automate the procedure of embryo selection.
   (c) To provide better culture conditions to the embryos by not disturbing them.
   (d) To simplify the daily workflow and reduce costs.

2. What were the most significant parameters of Meseguer's algorithm?

3. What are the main advantages of EmbryoScope™ Plus?

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Predicting Embryo Developmental Potential and Viability Using Automated Time-Lapse Analysis (Eeva™ Test)

Lei Tan, Alice A. Chen, and Shehua Shen

Note: The *Eeva*™ Test is CE mark approved in Europe and is cleared by the US FDA to provide adjunctive information on early human embryos during IVF cycles.

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Learning Objectives

- Limitations of traditional embryo assessment methods based on embryo morphology
- Emerging noninvasive technologies for embryo assessment
- Validation steps needed to introduce new tools and technologies into the IVF laboratories
- Automated time-lapse analysis in the Eeva Test
- Clinical outcome studies using the Eeva Test

42.1 Introduction

In in vitro fertilization (IVF) practice, technologies or tests that can help assess the viability and developmental competence of embryos may not only improve IVF success rates; they may also enable broader adoption of single embryo transfer (SET), which would in turn reduce multiple gestation pregnancies and lower associated maternal/fetal risks [1]. Currently, standard embryo assessment methods depend on morphological evaluation by microscope at a few static points in time (e.g., Day 1, Day 2, Day 3, and Day 5 post-insemination) [2, 3]. Morphological evaluation and selection, however, have lacked underlying biological understanding and sufficient predictability for implantation due to the inherent subjectivity of morphological grading [4–6] and the weak correlation between morphology and embryonic health [7–9]. Without predictive and objective tools that can supplement morphology, clinical embryologists are routinely forced to make their best guess of which embryo(s) to transfer among morphologically similar embryos.

New research in embryo assessment promises to provide additional information to help discriminate among morphologically similar embryos and improve the likelihood of selecting the single embryo that will successfully develop, implant, and result in a live birth. However, before new tools and technologies can be introduced into the IVF laboratory, several validation steps should be taken to ensure their safety, accuracy, and efficacy. These steps, which follow the initial discovery and development phases, may include biological validation, clinical validation, performance characterization, and comparison to standard of care [10]. Increasingly, clinical practitioners and patients also expect successful validation to result in approval from a regulatory body [11]. In this case, the regulatory body would require the intended use of the new tool or technology to be clearly stated and validated.

The objective of this chapter is to describe the development, validation, and practical application of the first noninvasive embryo viability assessment tool that has been designed to meet clinical test criteria and proven to add critical information to the decision-making practices of clinical embryologists. In describing this novel test, we review the criteria for development and validation of a clinical test, assess the scientific underpinning of prediction using time-lapse imaging, and introduce new advances in automation enabled by state-of-the-art computer vision software.

42.2 Clinical Testing

The goal of clinical testing is to help clinicians improve assessment or treatment decisions by determining a disease risk probability (in the case of a screening test) or diagnosing a disease (in the case of a diagnostic test) [12]. Typically, a clinical test is developed against a reference standard test that is more invasive, more risky, or prohibitively expensive. A clinical test may also be developed as a first-pass surrogate for a more invasive procedure (e.g., venous ultrasonography serves as a surrogate marker of vein thrombosis and reduces the need for a highly invasive venography procedure [13]; cell-free fetal DNA analysis of maternal blood can detect fetal genetic anomalies without requiring invasive amniocentesis or chorionic villus sampling procedures [14]).

In clinical embryology, several noninvasive candidate technologies have been proposed as surrogates for invasive embryo assessment and predictors of embryo developmental potential, including culture media assessment [15–17], cumulus cell assessment [18, 19], and time-lapse imaging [20–22]. Parallel advances in assay and embryo biopsy techniques have resulted in increased use of preimplantation genetic testing (PGT) together with blastocyst culture for assessing not only the genetic and chromosomal status of an embryo but also its overall implantation potential [23, 24]. While embryo biopsy is invasive to the embryo and typically relevant for IVF patients indicating a need for PGT, noninvasive technologies have the potential to be more economical and more broadly applicable. However, most noninvasive technologies are in early development stages; as such, they have not been prospectively validated in independent clinical trials and lack the basic prerequisites for successful clinical translation.

Three major requirements are needed in order to successfully implement a novel embryo assessment technology or test into the IVF laboratory. First, a predictive biomarker based on scientific evidence and validated in clinical studies must be associated with a confirmable and desirable outcome. Second, the detection of the predictive biomarker must be accurate, reliable, and practical to use by clinicians. Finally, the combined test (predictive biomarker + detection method) must be characterized for performance and shown to provide actionable information that improves the standard of care. In addition to these major requirements, scientists have increasingly demanded that new technologies be brought to patients via an evidence-based approach that includes basic research, which provides scientific understanding and clinical confidence in the test [10, 11, 22]. In a recent review, Palmer et al. noted that in reproductive medicine, it is common for only the first milestone—report of a novel biomarker correlation—to be achieved; whereas, successful culmination of all milestones ideally results in content for regulatory submission documents and eventual regulatory approval [11]. In support of this approach, new standards for reporting diagnostic accuracy (STARD) are being required of authors seeking to publish new test results [25].

42.3 The Eeva Test

The *Eeva™* Test was designed with clinical test criteria in mind and combines both a highly robust predictive marker of embryo development *and* a novel detection assay to rapidly obtain quantitative measurements of the biomarker in a clinical setting. Below we detail the use of Eeva in the IVF laboratory workflow and its underlying science and technology. We further describe clinical results from multiple prospective, multicenter studies that compare the use of Eeva results combined with morphology (adjunctive grading method) against morphology alone (traditional morphology grading method).

42.4 The Eeva Test Deployed in Standard Incubators or Benchtop Incubators

The original Eeva Test consisted of (1) a multi-well dish that provides individual culture for embryos with the same media drop, (2) an imaging system that collects data inside a standard incubator, and (3) intelligent software that automatically analyzes and measures cell division timings (Fig. 42.1).

Recently, other incubator formats have been developed and adapted for use with the *Eeva™* Test. Benchtop incubators are now being fitted with cameras, lighting sources, and time-lapse imaging software so that benchtop incubator users may also leverage Eeva's unique automation capabilities. Importantly, these new time-lapse benchtop incubators utilize both brightfield and darkfield imaging. Utilizing both brightfield and darkfield modalities together provides more information about embryos than brightfield alone and may lead to further discovery of novel features correlated to embryos' development potential.

42.5 Predictive Information Enabled by Time-Lapse

The first major requirement of a clinical test is identifying a predictive biomarker with correlation to a measureable, clinical outcome. Recently, increasing availability and usage of time-lapse imaging systems—both homemade and commercial variations—have enabled researchers to identify potential image biomarkers during human embryo culture in a safe and noninvasive manner. Time-lapse systems currently vary in a number of ways, harboring variations in format/footprint (e.g., some are enclosed in stand-alone box formats [26, 27], while others integrate seamlessly with conventional incubators [28–31]), embryo culture environment (e.g., some require individual culture of embryos [27], while others enable group culture [28–31]), and mode of illumination (e.g., most offer brightfield imaging, while others offer

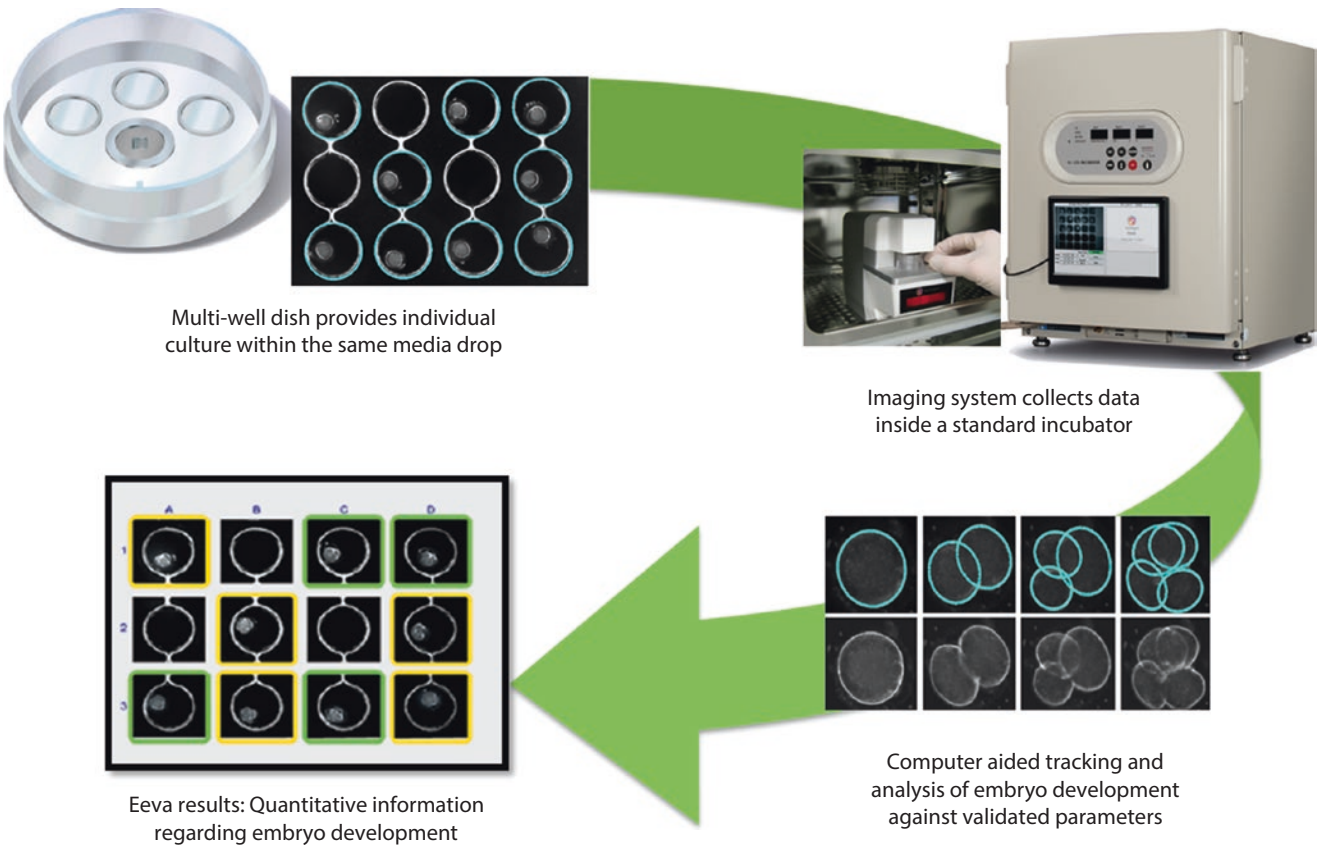


Fig. 42.1 The Eeva™ Test in the IVF laboratory workflow

darkfield or multimodal imaging). Importantly, all systems inherently have optical alignment, focusing, image capture, and image storage capabilities and utilize low-power illumination that exposes embryos to overall less light than conventional microscopy [27, 29, 31]. As a result of these advances, the clinical embryology field has been able to study the function and consequences of dynamics occurring during human embryo development and explore application to clinical practice.

Time-lapse imaging in clinical embryology has a relatively long history that can be broken down into two phases: an observational phase and a predictive phase. Beginning in 1997, time-lapse imaging was focused on observing new aspects of embryo development, such as polar body extrusion, fertilization, pronuclear formation and abuttal, cytoplasmic flares, and embryo hatching [26, 32–34]. Since 2010, time-lapse imaging has shifted focus to identifying predictive parameters that can help assess developmental outcomes of the embryo, such as blastocyst development, implantation, and most recently ploidy [27, 29, 30]. Numerous publications since 2010 have reported statistically significant correlations between time durations of cell stages and embryo outcomes. These studies, together with studies that have demonstrated safety of time-lapse and continuous monitoring of human embryos, are reviewed in detail elsewhere [20–22]. Overall, the increasing intensity of interest in time-lapse imaging suggests that noninvasive biomarker discovery is feasible, ongoing, and promising for use in the clinic.

Time-lapse biomarkers have great potential as predictive clinical tests, but the reproducibility of their predictive ability is critical to validating clinical utility. An analysis of the studies that defined a precise time window for embryo development prediction showed that the major predictors are biomarkers observed in early embryo development, before the 5-cell stage. In particular, the time from 2- to 3-cell (P2) and the time from 3- to 4-cell (P3) stage were reported in six out of seven of the studies that investigated which embryos are most likely to become a blastocyst or implant or be euploid [27, 29, 35–37] (Fig. 42.2) and several following reports [35, 38]. These P2 and P3 timings were consistent embryo viability predictors even when stimulation protocols [39], fertilization methods [40], culture media [41, 42], and culture environments [43] were varied. In addition to clinical reproducibility, mechanistic studies have also demonstrated that embryos with abnormal P2 and P3 timings exhibit distinct gene expression profiles [29], aneuploidy probabilities at

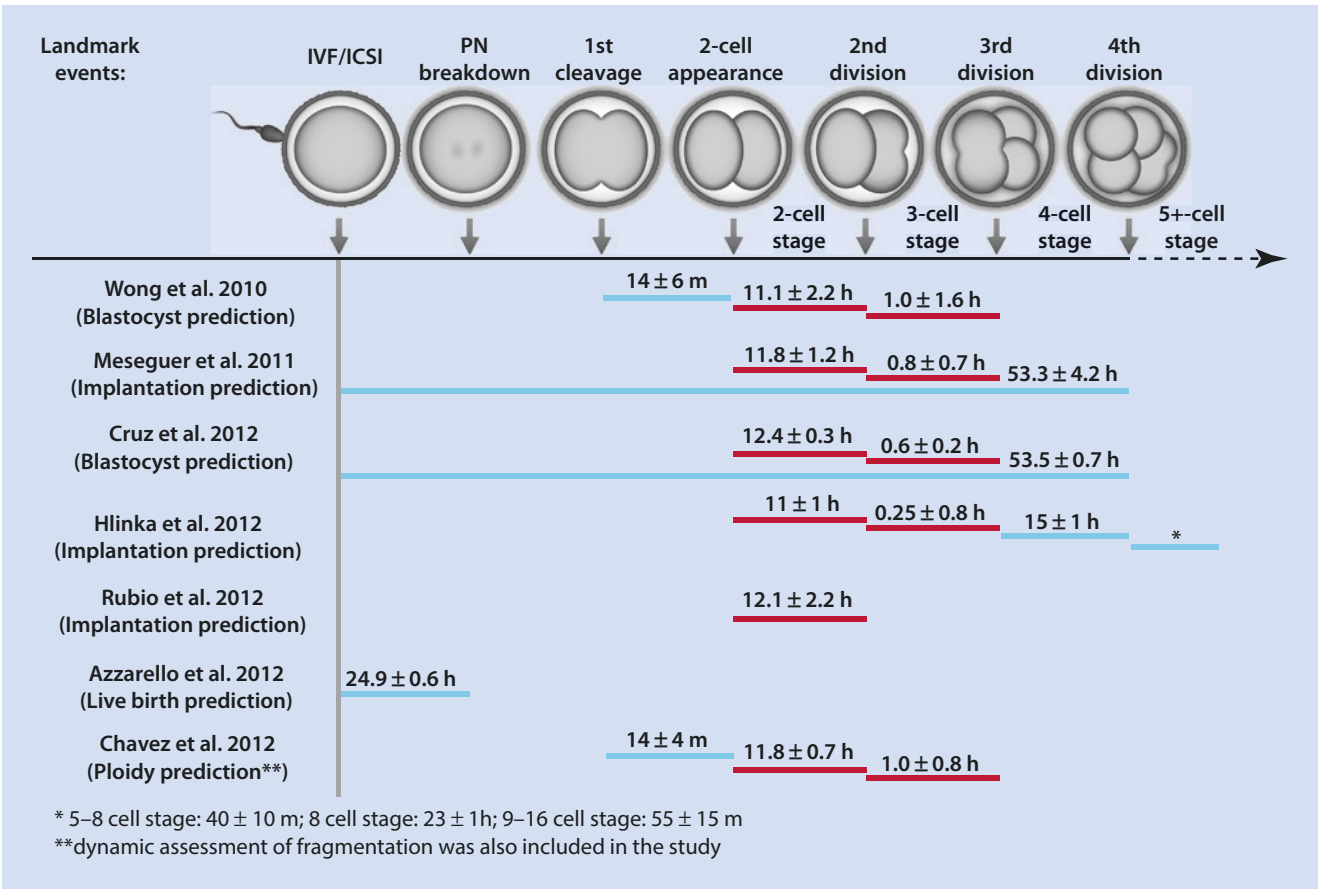


Fig. 42.2 Overview of published time-lapse biomarkers that are predictive of clinical outcomes. Landmark events captured by time-lapse imaging are mapped to the progression of preimplantation embryo development. Time-lapse biomarkers that have been used for prediction in at least three publications—and are implemented in the Eeva Test—are colored dark red, while others are *colored light blue*. Average values for embryo outcomes within the optimally predictive windows are labeled above *colored bars*. (Adapted with permission from Chen et al. [22])

the embryo level [30], aneuploidy at the single blastomere level [44], and micronuclei patterns [30], compared to embryos with normal P2 and P3 timings. This collection of evidence supports the conclusion that P2 and P3 are reliable across independent datasets, and, moreover, are grounded in basic scientific understanding. Development of the Eeva Test, therefore, was based on (1) incorporation of P2 and P3 into a simple classification tree and prediction algorithm and (2) development of intelligent image analysis software that could reliably measure P2 and P3 in a busy clinical laboratory.

42.6 Automation Enabled by Intelligent Software

The second major requirement of a clinical test is that it is technically reliable, accurate, and practical for daily laboratory use. Although time-lapse biomarkers may provide great clinical value, extraction of these markers requires manual assessment of hundreds of images per embryo, which would be prohibitively time-consuming and laborious for routine use. In order for predictive time-lapse biomarkers to be clinically realized, novel detection methods are required to automatically extract and quantify image data in a high-throughput fashion.

Computer vision software that can analyze embryo videos in real time is now transforming the time-lapse imaging field and allowing predictive biomarkers to be rapidly quantified in the clinical laboratory. Image analysis software is widespread in many areas of clinical laboratory medicine, such as oncology and pathology. In reproductive medicine, software tools for quantitative and rapid image analysis of sperm quality [45] and for screening cervical cytology samples [46, 47] are also available. Applied to clinical embryology, image analysis software is particularly challenging due to several unique features of the developing human embryo. Blastomeres of the human embryo may divide in multiple dimensions (e.g., in and out of plane) and may include confounding events (e.g., fragmentation, reverse cleavage, abnormal cleavage). In addition, for each patient, multiple embryos developing at variable rates must be surveyed.

In their 2010 report demonstrating the first use of three cell cycle parameters to predict embryo development, Wong et al. also established the first proof of concept of cell tracking software used to measure the cell cycle parameters in human embryos [29]. The Wong et al. approach utilized computer vision techniques to simulate images and compare the simulations to observed image data for 14 cryopreserved embryos. In their study, darkfield imaging was employed as it provided strong contrast of blastomere membranes for model estimations. The development of the Eeva Test introduced a data-driven probabilistic framework that uses approximate inference to quantify P2 and P3 and validated the Eeva Test's predictive performance for approximately 1000 fresh human embryos [31]. Eeva software predictions were shown to have good (>90%) agreement with manual predictions made by human observers [31]. Separate from this technological advance, other groups have performed research to help automate the morphological analysis of static embryo images to the blastocyst stage [48, 49] and the detection of cytoplasmic waves generated post-fertilization [50]. Since the objective in designing the Eeva Test was to provide embryologists with quantitative information about embryo development, the test focused on the clinically and scientifically validated predictive markers P2 and P3 and integrated automated software to quantify these validated parameters.

42.7 Clinical Outcomes for Using the Eeva Test

The final and often most elusive requirement for a novel clinical embryology test is that it is characterized for performance and shown to improve the standard of care. When performing a retrospective study, it is relatively straightforward to extract one or more promising biomarkers from a sampling of time-lapse videos. However, it is challenging and significantly less common to demonstrate comparable performance of the biomarker on an independent set of prospectively collected test data from multiple centers. It is rarer still that new potential selection methods are compared to traditional embryo selection methods based on morphology. Here, we describe how the Eeva Test provides validated and actionable information that may improve current embryo selection based on morphology.

42.7.1 The Eeva Test Is Independently Validated in Diverse Clinical Embryology Laboratories

Following the development of the integrated Eeva Test, we conducted a prospective, five-center clinical study that was designed to assess its performance on a diverse and independent clinical dataset and to evaluate the effectiveness and utility of using the test as an adjunct to traditional morphological assessment (► ClinicalTrials.gov #NCT01369446) [31]. Specifically, we sought to test the Eeva Test's ability to assist embryologists in selecting embryos that have the greatest potential to develop into usable blastocysts (i.e., blastocysts suitable for transfer or freezing) as well as the Eeva Test's impact on embryologists' embryo selection decisions. The ability to predict blastocyst formation by Day 3 has clinical value as it could potentially improve IVF success rates while avoiding the potential risks associated with extended culture for blastocyst transfer [51–56].

Our initial results demonstrating the predictive power of time-lapse biomarkers P2 and P3 for identifying embryos with high developmental competence were consistent with Wong et al. and other successive studies [29]. Using refined cutoffs of P2 9.33–11.45 h and P3 0–1.73 h, the Eeva Test automatically differentiated a usable blastocyst from an

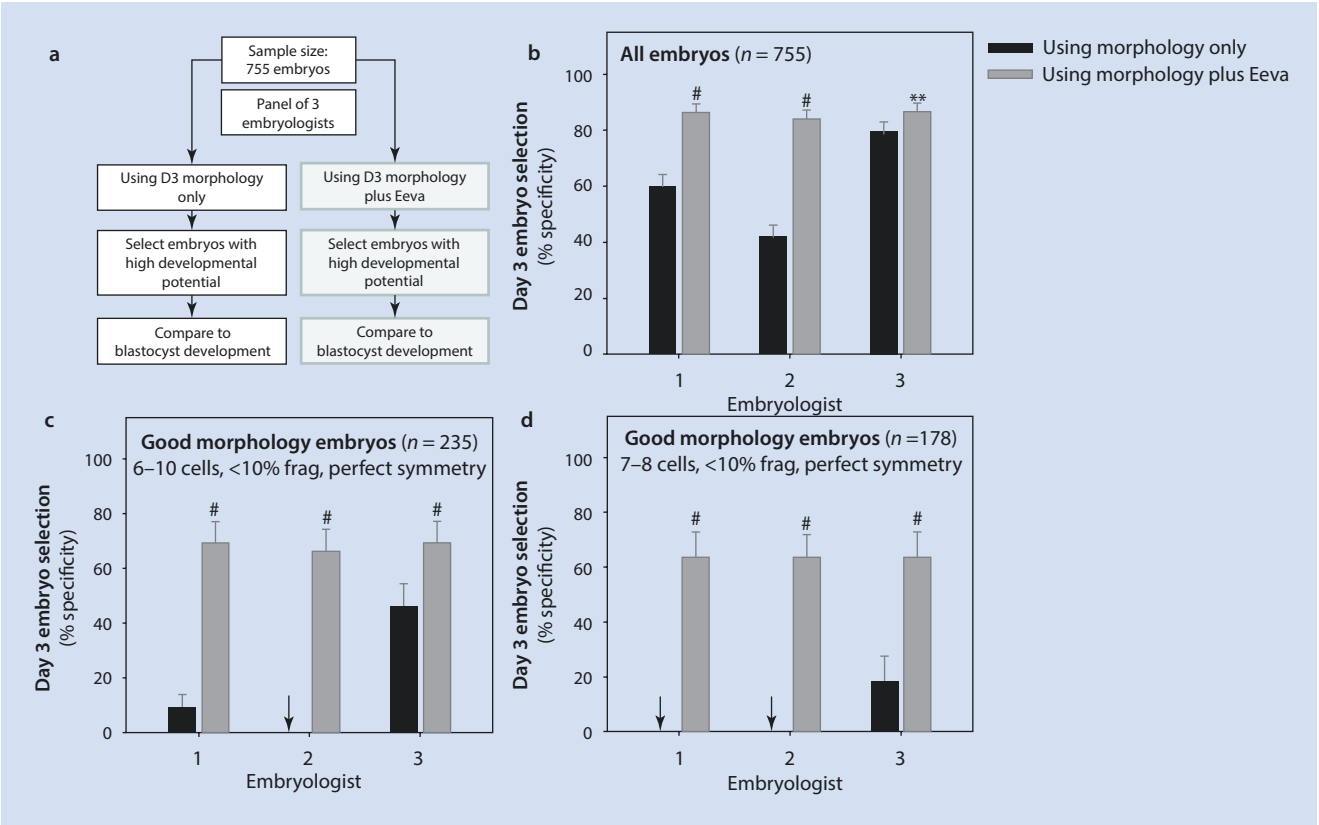


Fig. 42.3 Adjunctive use of D3 morphology plus Eeva Test results significantly improves the specificity of D3 embryo selection. **a** Design for an embryo selection cohort study comparing the use of D3 morphology vs. D3 morphology plus Eeva Test results. **b**–**d** D3 embryo selection by individual embryologists (1, 2, and 3) using morphology only versus morphology plus Eeva Test for **b** all embryos ($n = 755$); **c** "good morphology" embryos ($n = 235$), defined using criteria of 6–10 cells, <10% fragmentation, and perfect symmetry; and **d** "good morphology" embryos ($n = 178$), defined using more stringent criteria of 7–8 cells, <10% fragmentation, and perfect symmetry. Note that embryologists 1 and 2 were very conservative in their morphological assessments and expected that almost all D3 "good morphology" embryos would become usable blastocysts. Error bars represent upper 95% confidence interval. $^{**}p < 0.01$, $\#p < 0.0001$. (Adapted with permission from Conaghan et al. [31])

arrested embryo with significantly improved diagnostic specificity (85%) compared to traditional morphology (57%, $p < 0.0001$) [31]. Specificity, or the ability to correctly predict which embryos will arrest, is highly relevant to embryo selection since traditional morphology is most limited in selecting among "good morphology" embryos. Furthermore, development and validation of the Eeva Test were performed on clinical data collected from five IVF clinics undergoing their own standard procedures for stimulation, egg retrieval, embryo culture, and insemination, suggesting broad applicability of the Eeva Test in diverse clinical embryology laboratories.

42.7.2 The Eeva Test Improves Embryo Selection and Reduces Embryologist Variability

Since clinical embryologists are faced daily with the challenge of selecting from among several comparable embryos those which are the top candidates for transfer, it was important to assess the impact of the Eeva Test on embryologists' embryo assessment decisions. Our prospective study design evaluated the embryo assessment of three experienced clinical embryologists for 755 embryos. Two methods of embryo assessment were compared: in the first arm, embryologists made predictions about embryo development using Day 3 morphology only (traditional grading method); in the second arm, the same embryologists made predictions about embryo development using Day 3 morphology plus Eeva Test results (adjunctive grading method) (Fig. 42.3a).

When Eeva Test was used adjunctively with Day 3 morphology, each embryologist's likelihood of selecting embryos that would develop to blastocysts was significantly improved compared to traditional morphological methods alone (Fig. 42.3b). This improvement was even more pronounced among a subset of $n = 235$ embryos that were preselected as having "good" morphology (6–10 cells, <10% fragmentation, perfect symmetry) (Fig. 42.3c) and a subset of $n = 178$ embryos where "good" morphology was more stringently defined (7–8 cells, <10% fragmenta-

tion, perfect symmetry) (Fig. 42.3d). As expected, each case demonstrated remarkably high inter-individual variability when morphology alone was used for embryo assessment. When the Eeva Test was used together with morphology, the embryologists' performances were more consistent, as the standard deviation among embryologists was reduced [31].

In a separate study, we used the Eeva Test to assess differences in embryo scoring among a diverse set of embryologists, postulating that better, more consistent embryo scoring would positively impact any embryologist's ability to choose the embryo with the highest developmental potential [57]. This study was performed by looking at five different embryologists from unique practices around the United States, including three senior embryologists with at least 10 years of clinical experience and two junior embryologists with less than 3 years of experience from practices that performed between 300 and 1000 fresh IVF cycles per annum. To assess embryologists' performance, odds ratio (OR) and other diagnostic measures were calculated by comparing prediction results to true blastocyst outcomes.

When Eeva Test results were used adjunctively with morphology, the odds of an embryo forming a blastocyst was 3.51-fold (95% CI = 2.62–4.69) higher in the group predicted to develop into blastocysts than in other embryos. In contrast, the OR using morphology alone was 2.69 (95% CI = 2.06–3.50). This improvement in OR was also assessed in the subset of morphologically good and fair embryos. By morphology alone, the OR for this subset dropped to 1.68, slightly better than random prediction ($p < 0.0001$). Adding Eeva Test results improved OR to 2.57, a 53% increase over traditional morphology and significantly better than random prediction ($p < 0.0001$, Fig.42.4a). In addition to OR, Eeva Test results also helped improve the positive predictive value (PPV) over morphology alone (54% vs. 43%, $p = 0.02$) while maintaining the same level of negative predictive value (NPV, 68% vs. 68%, Fig. 42.4b).

These results indicate that the Eeva Test aids embryologists of diverse backgrounds by distinguishing among similar-looking embryos that are evaluated first by morphological criteria. Using Eeva Test results as an adjunct to morphology, every individual embryologist's prediction performance was improved (Fig. 42.4c). Using the Eeva Test adjunctively to morphology, the variability in performance across all five embryologists was reduced from a range of 1.06 (OR = 1.14–2.20) to a range of 0.45 (OR = 2.33–2.78, Fig. 42.4c). Notably, the embryologist with the greatest improvement in OR was one of the senior embryologists with more than 10 years of training in morphology grading. Since intra- and inter-operator variability in morphological grading has been shown to negatively impact IVF success rates [4, 6], adjunctive use of the Eeva Test may improve the standardization, reproducibility, and ultimate success of Day 3 embryo selection.

42.7.3 Eeva Test Results Correlate to Implantation and Pregnancy

While the first critical steps in developing the Eeva Test as a predictive and automated clinical assay have been achieved, further work is needed to confirm the impact of Eeva Test results on implantation and pregnancy outcomes. Toward this goal, we performed the first analysis examining whether the time-lapse markers used by the Eeva Test correlate with implantation and pregnancy outcomes. We focused our analysis on embryos that were transferred at the blastocyst stage using standard morphological selection criteria (without Eeva Test results). Of 141 embryos transferred at the blastocyst stage, those embryos denoted by the Eeva Test as having a high probability to develop (Eeva High indicating P2, 9.33–11.45 h, and P3, 0–1.73 h) had a statistically higher chance of implantation than those embryos denoted by the Eeva Test as having a low probability to develop (Eeva Low indicated by P2 or P3 out of specific timing windows) (49% vs. 21%, $p < 0.001$) (Fig. 42.5). Similarly, for these 77 blastocyst transfer patients, those with at least one Eeva High embryo transferred were more likely to achieve clinical pregnancy (60% vs. 40%, $p = 0.09$) and ongoing pregnancy (56% vs. 37%, $p = 0.11$) than those with only Eeva Low embryos transferred. These results add further evidence to recent retrospective reports showing correlation between P2, P3, and implantation [27] and pregnancy outcomes [58].

The Eeva Test's fully automated algorithm was further examined in a blinded, multicenter study published in 2014 [59]. This study took place in six different IVF centers, each recruiting and treating patients using their own protocols, laboratory equipment, and consumables. A total of 331 transferred embryos with known implantation from 205 patients enrolled at 6 IVF clinics were analyzed. We found that Eeva High embryos had a significantly higher probability of successful implantation (37%, 41/111) than Eeva Low embryos (23%, 50/220, $p = 0.003$, Fig. 42.6a). Eeva Test results were also correlated with clinical pregnancy rates. Patients were divided into two groups: patients with at least one Eeva High embryo transferred and those with no Eeva High embryo transferred. Patients' clinical characteristics for the two groups were compared, including egg age, number of eggs retrieved, number of 2PNs on Day 1 and number of embryos transferred. There was no statistically significant difference found for any of the clinical characteristics assessed. However, patients with at least one Eeva High embryo transferred had significantly higher clinical pregnancy rates than those with no Eeva High embryos transferred (51% vs. 39%, $p = 0.04$). Additional analysis of embryo implantation revealed a statistically significant difference between the two groups of patients (34% vs. 25%, $p = 0.03$). In a three-category version of the Eeva Test results (High/Medium/Low), Eeva High embryos had the highest likelihood of implantation (37%), followed by Eeva Medium

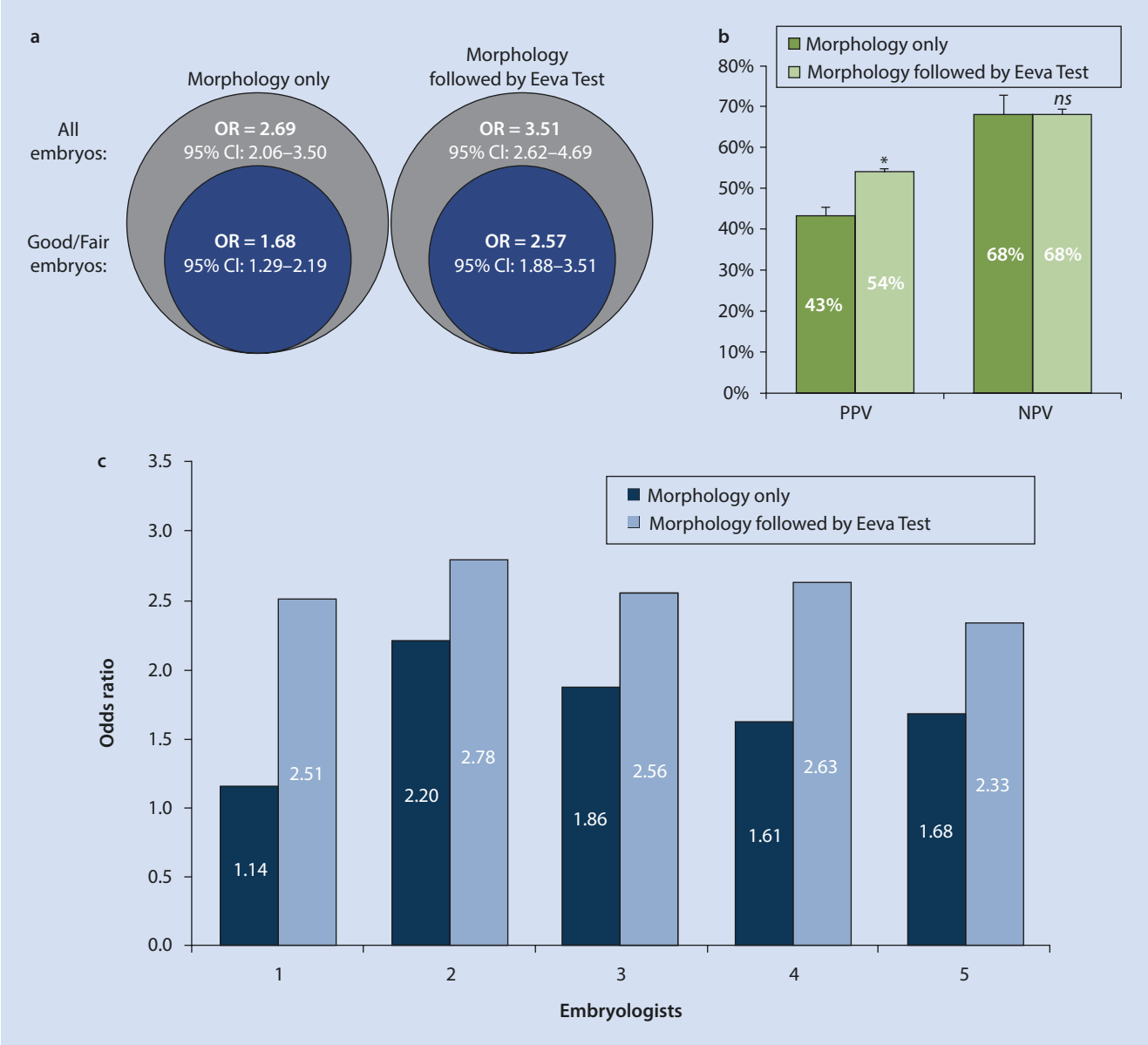


Fig. 42.4 Adjunctive use of Day 3 morphology plus Eeva Test results significantly improves Day 3 embryo selection and consistency among embryologists. **a** OR for predicting blastocyst formation using Morphology Only (left) and Morphology followed by Eeva Test (right). ORs and 95% confidence intervals were calculated for all embryos (represented in gray) and for the subset of embryos graded as good/fair (represented in blue). **b** Mean PPV and mean NPV across all embryologists predicting blastocyst formation using Morphology Only and Morphology followed by Eeva Test, among good/fair embryos. *$p = 0.02$, ns (not significant; error bars represent upper 95% confidence intervals). **c** Consistent improvement in ORs for individual embryologists who predicted blastocyst formation using Morphology Only and Morphology followed by Eeva Test, among good/fair embryos. (Reproduced with permission from Diamond et al. [57])

(35%) and Eeva Low (15%); and the difference in implantation rates between Eeva High vs. Eeva Low embryos and Eeva Medium vs. Eeva Low embryos was statistically significant ($p < 0.0001$, $p = 0.0004$, respectively, Fig. 42.6b). Furthermore, although the IVF centers each followed their own standard procedures for embryo culture and selection, Eeva Test results correlated to embryo implantation consistently across the different IVF centers (Fig. 42.6c).

Consistent with our study results, a growing body of published, independent studies have reported that Eeva Test results correlate to implantation and/or pregnancy regardless of patient population or IVF practice patterns. Kirkegaard and colleagues performed a retrospective multicenter analysis of 1519 transferred embryos from 7 clinics located in 3 countries and found that embryos manually analyzed and classified as High had a statistically significantly higher implantation rate (30% relative increase) than embryos that were manually classified as Low [60]. Kieslinger and colleagues showed that in good prognosis patients (<41 years old, <3 previous attempts, and ≥ 5 2PNs) with mostly SET,

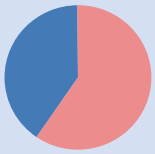
Patient population	# Pts	# Embs	Avg age (years)	Implantation rate	Clinical pregnancy rate	Ongoing pregnancy rate
At least 1 Eeva High transferred	47	89	32.1 ± 5.2	**49%** (44/89) 	**60%** (28/47) 	**55%** (26/47) 
Only Eeva Lows transferred	30	52	32.2 ± 5.1	**21%** (11/52) 	**40%** (12/30) 	**37%** (11/30) 
p-value			*p = 0.9*	*p<0.001*	*p = 0.09*	*p = 0.11*

Fig. 42.5 Time-lapse markers used by the Eeva Test are correlated with positive implantation and pregnancy outcomes. A retrospective analysis of implantation and pregnancy rates was performed for two populations of patients with Eeva imaging and blastocyst transfer based on morphology only. The two patient populations included those with at least one Eeva High transferred (i.e., one Eeva High embryo could be transferred with another Eeva High or Eeva Low embryo) and those with only Eeva Low transferred (i.e., one or more Eeva Low embryos were transferred). The Eeva High vs. Low denominations are based on whether time-lapse markers P2 and P3 are within defined time windows (P2, 9.33–11.45 h, and P3, 0–1.73 h). (Adapted with permission from Chen et al. [22])

transferring Eeva High and Medium embryos resulted in a significantly higher ongoing pregnancy rate of 36.8% (89/242) compared to 18.4% (7/ 38) for Eeva Low embryos (p = 0.02) [61]. Aparicio-Ruiz and colleagues conducted a multicenter retrospective study of 626 IVF cycles using donor oocytes with the following findings: (1) Eeva Test results were correlated with embryo implantation in not only cleavage-stage embryo transfer but also blastocyst transfer (implantation rates for cleavage stage transfer: Eeva High 38.2%, Medium 31.7%, and Low 26.1%; implantation rates for blastocyst transfer: Eeva High 66.7%, Medium 50%, Low 31%); (2) Patients where no Eeva High embryos were transferred had an ongoing pregnancy rate of 46.7%, while patients where at least one Eeva High embryo was transferred experienced a significantly increased ongoing pregnancy rate of 67% [62]. In comparison, no significant difference was observed for ongoing pregnancy rates in patients with or without morphology grade A embryos transferred (60.2% vs. 59.3%). This broad collection of publications by independent investigators provides strong evidence that the Eeva Test differentiates embryos with higher implantation potential, and may therefore complement traditional morphology to aid in embryo assessment.

42.7.4 The Eeva Test Improves Clinical Outcomes

The gold standard for evaluating the effectiveness of time-lapse-based embryo assessment is a well-designed randomized controlled trial (RCT). To ensure that an RCT is adequately powered, pilot studies are needed to estimate the sample size for RCT. Adamson and colleagues assessed the impact of using the Eeva Test in adjunct with traditional morphology by conducting a prospective concurrent cohort-controlled pilot study [63]. A total of 319 patients were assessed, 98 in the test group (the Eeva Test was used in embryo selection) and 221 in the control group (traditional morphology alone was used for embryo selection). All patients met the same inclusion/exclusion criteria including <41 years of age at the start of the IVF cycle, planned day 3 transfer, fewer than three failed IVF cycles, at least four zygotes (2PN on Day 1), and no PGT. Implantation rate and clinical pregnancy rate were significantly higher in the test group in which the Eeva Test was used compared to the control group (implantation rate, 30% vs. 19%, p = 0.003; clinical pregnancy rate 46% vs. 32%, p = 0.02, Fig. 42.7a, b). In a sub-analysis of the test group, the authors found that patients receiving at

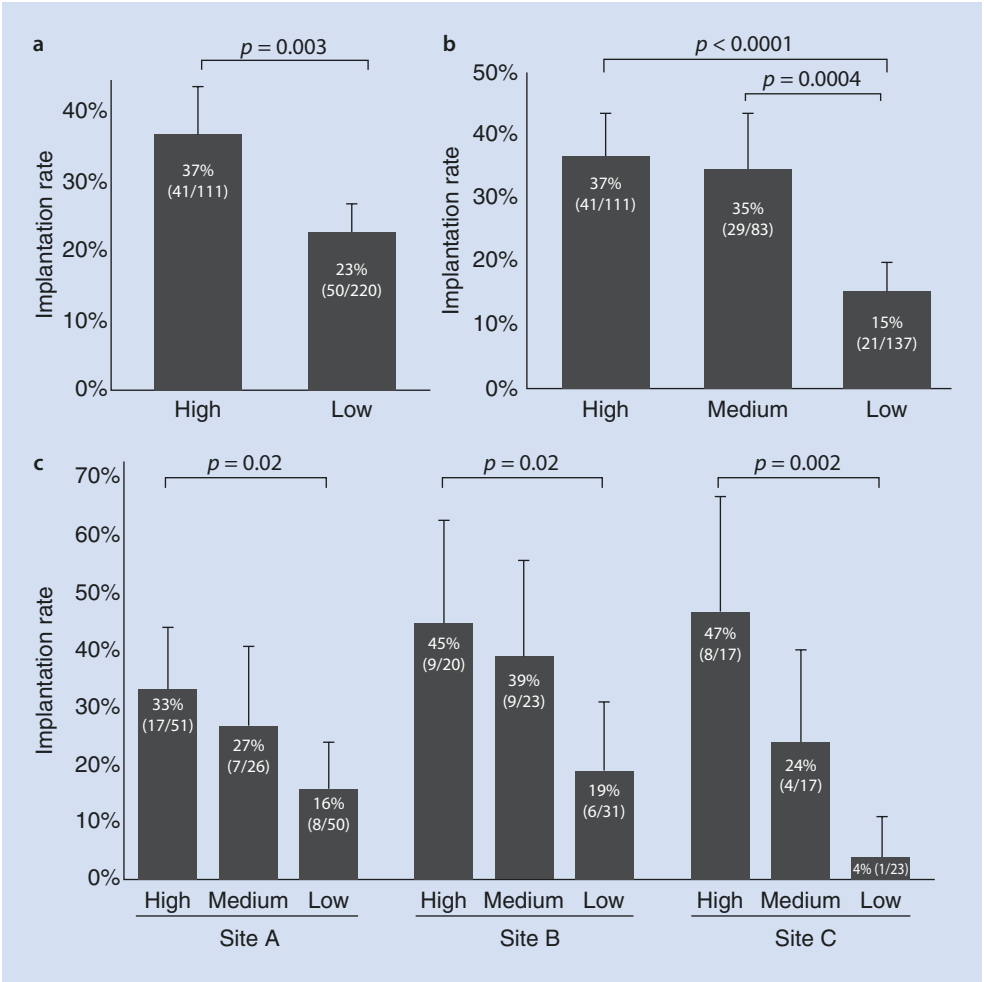


Fig. 42.6 The Eeva Test results are correlated with implantation. **a** Implantation rates for Eeva two-category High versus Low scored embryos. The difference in implantation rates between Eeva High and Eeva Low embryos is statistically significant: $P = 0.003$. **b** Implantation rates for Eeva three-category High, Medium, and Low scored embryos. Implantation rates between High versus Low and Medium versus Low were significantly different ($P < 0.0001$ and $P = 0.0004$, respectively). **c** Implantation rates for embryos with Eeva High, Medium, and Low scores from three clinical sites with at least 50 embryos of known implantation data per site. For all three sites, the difference in implantation rates between Eeva High and Low embryos is statistically significant (chi-squared test): $P = 0.02$ (site A); $P = 0.02$ (site B); $P = 0.002$ (site C). Error bars represent 95% upper confidence limit. (Reproduced with permission from VerMilyea et al. [59])

least one Eeva High embryo had significantly higher implantation rates than patients receiving only Eeva Low embryos (36.8% vs. 20.6%, Fig. 42.7c). Eeva High embryos compared with Low embryos also had significantly higher implantation rates (44.7% vs. 20.5%, Fig. 42.7d). Among morphologically good or top embryos, Eeva High embryos were also more likely to implant than Eeva Low embryos (Fig. 42.7e–f). These results demonstrated the clinical impact of the Eeva Test in patients receiving Day 3 embryo transfers, adding further evidence that the Eeva Test results add valuable information beyond traditional morphology.

42.8 Conclusion and Future Work

The Eeva Test is the first time-lapse-based clinical test for embryologists that has been cleared by the US Food and Drug Administration to aid in embryo selection. The Eeva Test has been validated in a series of prospective, multicenter studies and shown to add value to embryologists' morphological evaluations by improving embryo assessment and reducing variability among embryologists. It combines both robust, predictive markers of embryo development along with a novel automated detection assay to rapidly obtain quantitative measurements of early embryo development markers that have been proven to be of value in determining the best embryo in a cohort of multiple embryos. The Eeva Test meets several important prerequisites for implementation in clinical practice: it is scientifically and clinically validated, it considers a practical intended use model, and it has demonstrated clinical utility compared to the standard of care.

As the Eeva Test continues to be developed in the lab, new markers of embryo viability and/or implantation potential can be unearthed. Additionally, as more labs routinely grow embryos to the blastocyst stage, significantly more frames of videos may be assessed to learn more about embryo develop-

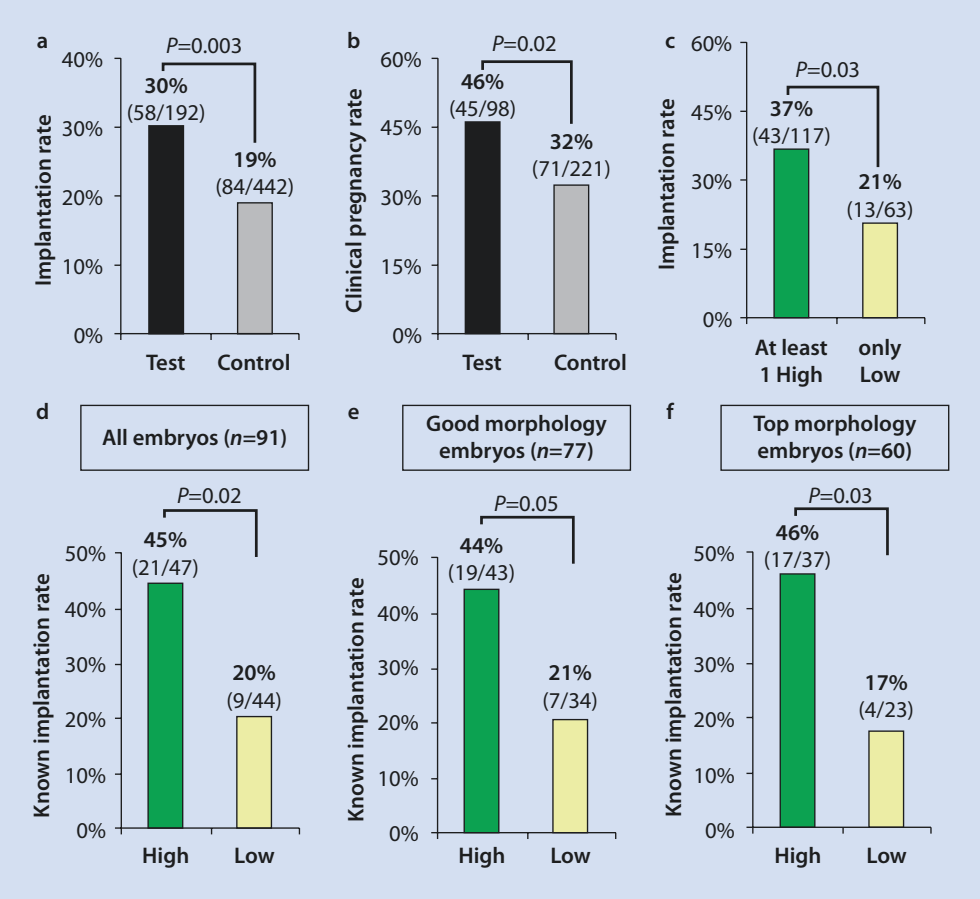


Fig. 42.7 Using Eeva results together with morphology improves implantation and pregnancy rates for Day 3 embryo transfer. **a** Implantation rates in embryos selected using morphology plus Eeva results (Test) versus using morphology alone (Control). **b** Clinical pregnancy rates in patients whose embryos were selected using morphology plus Eeva results (Test) versus using morphology alone (Control). **c** Implantation rates for patients who had at least one Eeva High embryo transferred versus those who had only Low embryos transferred. **d**–**f** Known implantation rates for Eeva High versus Low embryos in all transferred embryos **d**, all good-quality embryos **e**, and top-quality embryos **f**. (Reproduced with permission from Adamson et al. [63])

ment. Invasive tests of embryo chromosomal abnormality, for instance, PGT, continue to be applied to more embryos around the world, which supplies further outcome data to aid in ongoing time-lapse research. Ongoing time-lapse research will assess how noninvasive time-lapse imaging may be used in conjunction with invasive tests such as PGT to further improve embryo assessment. We envision that future versions of the Eeva Test will incorporate complementary morphological features and timings, blastocyst stage milestones, and other prospectively validated parameters. We further expect that automated analysis of these more complex morphological and timing features will require increasingly sophisticated approaches and likely a combination of illumination techniques. The introduction of any automated algorithm will be accompanied with the full spectrum of clinical validation steps described here to ensure safety and efficacy for clinical use.

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Review Questions

1. What are the limitations of traditional embryo assessment methods based on embryo morphology?
2. What are the emerging noninvasive technologies for embryo assessment?
3. What is the information provided by time-lapse imaging?
4. What are the validation steps needed before new technologies are introduced?
5. What types of evidence support the use of the Eeva Test?
6. What are some of the future directions for time-lapse-based embryo assessment tool?

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Proteomics and Metabolomics

Manesh Kumar Panner Selvam, Damayanthi Durairajanayagam, and Ashok Agarwal

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Learning Objectives

- Different proteomic and metabolomic techniques currently applied in practice
- Proteomic and metabolomic biomarkers for assessing embryo quality
- Predictive model developed based on metabolomic technique for embryo viability
- Secretome/spent culture media as a source for embryo quality assessment
- Oocyte and embryo selection using the omics platform

43.1 Introduction

Embryo grading and selection are the most essential steps involved in assisted reproductive technology (ART) prior to embryo transfer (ET). Unfortunately, not all ETs will result in successful implantation or establish a pregnancy as ~70% of embryos transferred will fail to implant and the pregnancy rate for each ET cycle is only 35.5% [1, 2]. The development of advanced embryo hatching techniques such as laser-assisted hatching (LAH) has been able to increase clinical pregnancy and embryo implantation rates, but has failed to decrease miscarriage and stillbirth rates [3]. Therefore, successful ET and implantation depend not only on factors such as ET technique and endometrial receptivity but also on the quality of embryo used in the ET procedure. In IVF practice, multiple ETs are performed in a single cycle to increase pregnancy rates. However, multiple pregnancy due to ART is considered as a significant health issue for pregnant women that could result in preeclampsia, heart failure, and pulmonary edema [4], while in infants it is associated with the risk of premature birth and physical disability [5]. Therefore, single embryo transfer (SET) is advised to minimize the risk of multiple pregnancies to avoid the associated health complications and is achieved by selection of high-quality embryos with high implantation potential [6].

In general, the conventional technique for grading and selection of viable embryos relies on microscopic evaluation and real-time morphological assessment of embryos [7]. This simple approach mainly determines cleavage and blastocyst formation in a subjective manner, which is inadequate for the prediction of embryo quality. Poor understanding of the molecular biology and processes involved in embryo development is among the limitations in distinguishing viable embryos [8]. Even though the traditional means of morphological assessment of embryo quality is inexpensive and used extensively in clinical practice, its prediction power is moderate for embryo implantation with low inter- and intra-observer variations [9]. The prolonged or extended in vitro culture of embryos also has increased risk of epigenetic alterations [10, 11]. Hence, non-morphological parameters have to be accounted for as well during the selection of embryos that are of superior quality. Techniques such as fluorescent in situ hybridization (FISH) [12, 13], comparative genetic hybridization (CGH), and single nucleotide polymorphism (SNP) arrays [14, 15] have been used successfully for preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS) to assess the nuclear quality of the embryo. However, analysis of genetic material using these techniques would mean that the embryos are subjected to invasive biopsy methods. As such, the use of noninvasive techniques for identification of biomarkers in assessing embryo quality could potentially overcome the abovementioned hurdles to improve ART outcomes.

The high-throughput platforms proteomics and metabolomics are two promising noninvasive techniques for studying biomarkers at a molecular level for selection of high-quality embryos. In-depth analysis of proteins and their post-translational modifications (PTMs), as well as metabolites secreted by embryo into the culture media, provides valuable information about key biomolecules that could help differentiate normal embryos from abnormal ones (Fig. 43.1).

43.2 Proteomics and Embryo

Proteomics is the quantitative and qualitative study of the cell or tissue proteome. New-generation technologies have enabled the identification of thousands of proteins in a short period of time using shotgun or bottom-up proteomic approaches. In the field of ART, proteomic tools can be used efficiently either for prognosis or diagnosis, using potential protein biomarkers to improve pregnancy rates. Proteomics is heavily dependent on bioinformatics tools to simplify the complicated raw data. Innovative global proteomic approaches are able to identify the complete protein profiles of the embryonic secretome at different developmental stages of the embryo. Apart from this, additional information about PTMs and interactions between proteins could explain the complex biological and molecular pathways regulating embryo development [16].

Numerous studies have reported the involvement of proteins during different stages of oogenesis, embryo implantation, and pregnancy maintenance using modern proteomic techniques such as MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) and SELDI-TOF (surface-enhanced laser desorption/ionization time-of-flight) that combine the precision of mass spectrometry (MS) with high-throughput protein microarrays [17, 18]. As the expression of these proteins differs from normal to abnormal cells and even between the same cells at different time periods based on their function, protein profiling would help in detecting malfunctioning embryonic proteins. Also, the expressions of specific protein are altered in endometriosis, polycystic ovary syndrome (PCOS), and preeclampsia, which can affect oocyte maturation and embryo implantation [19–22].

Therefore, proteomic approaches are widely recommended for the identification of embryonic protein biomarkers, which can be used as noninvasive techniques to access embryo quality. This will aid in single embryo selection during assisted reproductive techniques to decrease unwanted multiple gestation as well as failed ART procedures.

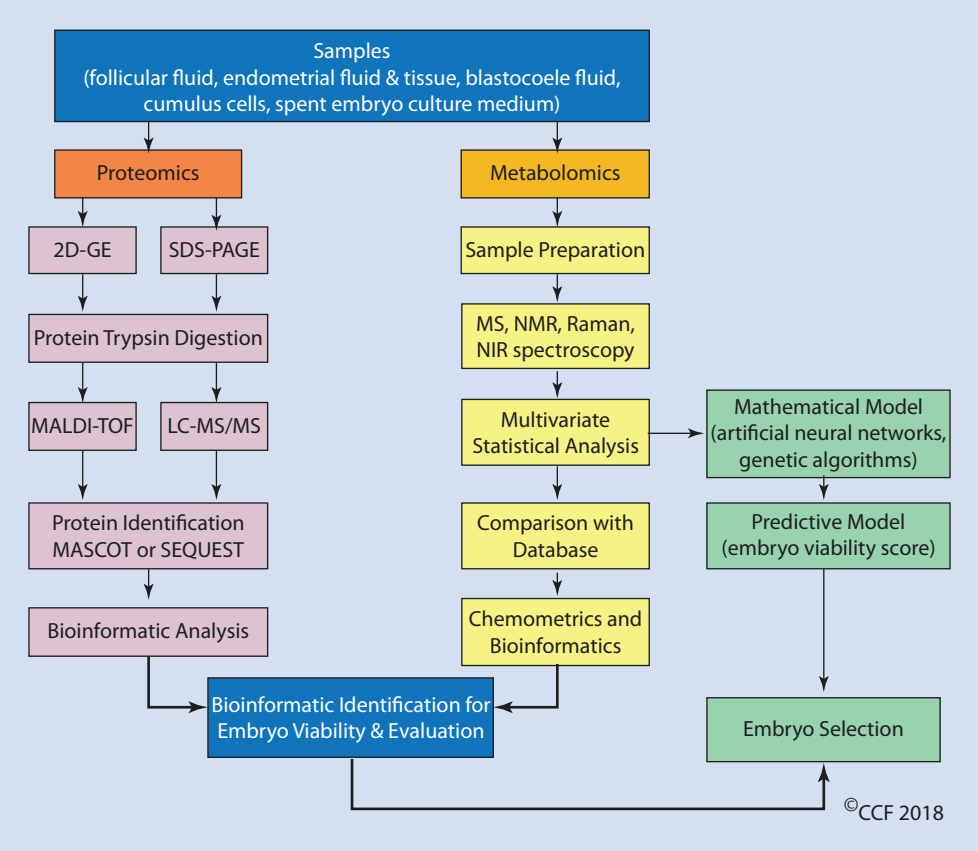

Fig. 43.1 Proteomic and metabolomic pipeline for embryo evaluation and selection

43.3 Different Proteomic Techniques

Basically, proteomic techniques are categorized into separation and identification techniques. Their usage differs between laboratories based on experimental requirements. Current proteomic approaches are aimed at protein profiling, differential expression of proteins, localization and identification of PTMs, analysis of protein-protein interactions, and protein involvement in regulating biological and molecular pathways. The extracted proteins are initially either subjected to 1D or 2D electrophoresis followed by MS to analyze the proteome. Furthermore, these identified proteins are validated or conformed by western blotting (Fig. 43.1).

43.3.1 Two-Dimensional (2D) Gel Electrophoresis

2D-elecrophoresis is the most widely used technique in proteomic research for resolving proteins. Proteins are separated according to their isoelectric focusing (IEF) property and molecular weight (MW) on SDS-PAGE. Proteins resolved on the gel are visualized by staining the proteins and then quantified based on the intensity of the spots on the gel. A modified version of the 2D-gel electrophoresis known as difference gel electrophoresis (DIGE) is used to identify differentially expressed proteins (DEPs). Two samples (control and test) are labeled separately with fluorescent cyanine (Cy) dyes (Cy3 and Cy5) and electrophoresed together on the same 2D-gel. The fluorescent signals generated by the same protein (with identical IEF and MW) present in two different samples are analyzed using the image analysis software program for relative quantification of the DEPs. DIGE is a high-sensitive technique and can even detect proteins with a 10% difference in their expression [23].

43.3.2 Mass Spectrometry

Mass spectrometry (MS) is an important and highly reliable high-throughput technology in the field of proteomic research that is utilized to identify the proteins and polypeptides based on their ion mass/charge ratio (m/z). Mass spectrometry can analyze thousands of peptides and proteins in an unbiased, systematic manner [24]. Tandem MS (MS/MS) further provides information about the specific ions. MS-based proteomics is used for protein identification, protein sequencing, identification of PTMs, and characterization of multi-protein complexes. Prior to injecting the sample into the MS instrument, they are subject to either gel-based or gel-free proteomic approaches to separate the proteins. Gel-based techniques include separation of proteins using 1D electrophoresis, then each lane of gel is excised into small parts. The proteins present in the gel are subjected to "in-gel" proteolytic digestion with trypsin. Furthermore, these proteins are eluted from the gel and analyzed using MS [23].

In the gel-free approach, liquid chromatography (LC) or gas chromatography (GC) is applied for the separation of polypeptides. MS technology can measure the mass of large intact proteins (>100 kDa) and small peptides obtained after proteolytic digestion. Incorporation of such advanced analytical separation techniques (LC, GC, high-performance liquid chromatography (HPLC), and capillary electrophoresis) reduces the complexity of the protein samples analyzed in MS.

43.3.3 Bioinformatics Analysis of MS Data

The molecular masses of peptides obtained from MS is compared with previously sequenced proteins, expressed sequence tags (EST), and DNA sequences available in a global database. Protein analysis software applications such as Mascot, SEQUEST, and X! Tandem are utilized to determine the spectral counts, whereby each program works on specific algorithms to generate a list of proteins. Gene names corresponding to the identified proteins are provided for functional annotations, while gene prediction programs are used to identify the proteins unavailable in the database based on their functional group [25]. The next step for the identified list of proteins is gene ontology (GO) analysis using databases such as GO Term Finder and GO Term Mapper, which provide information about the proteins based on their function, localization, structure, and biological function in cellular pathways. The genes/proteins are basically classified into cellular components, biological processes, and molecular functions. Interaction network and pathway analysis for the group of proteins are performed using proprietary software packages such as Ingenuity Pathway Analysis (IPA) and Metacore™ to identify the pathways, interactions, and cellular distribution of the proteins. An online bioinformatics tool, Search Tool for the Retrieval of Interacting Genes/Proteins (STRING), displays the functional link in protein-protein interactions [26].

43.4 Proteome of Follicular Fluid

Proteome reflects both internal and external environmental status. Follicular fluid (FF) provides the microenvironment for the development of oocytes. It determines the quality of the oocyte and also the fertilization potential with spermatozoa and embryo development [27]. FF is an easily available, noninvasive biological material that is highly suitable for proteomic studies. In general, during ovum pick up or retrieval, FF is also aspirated together with the oocytes. As it is enriched with proteins, FF serves as good medium for identification of potential biomarkers to assess oocyte quality and ART outcome.

Proteomic profiling of FF began 20 years ago, when 2D-gel electrophoresis was used to define the proteins present in the FF [28]. Preliminary FF proteomics suggested that follicular maturation is an inflammatory process that is regulated by various acute phase proteins and antioxidant proteins (such as catalase, superoxidase, and HSP27) [29]. Furthermore, Twigt et al. identified 246 proteins in FF, of which the majority were associated with coagulation and the inflammatory reaction [30]. Comprehensive data mining and bioinformatics analysis of 617 unique FF proteins revealed that metalloproteinases, thrombin, and vitamin-D receptor/retinoid-X-receptor-alpha can be used as biomarkers for assessing ovarian health and oocyte quality [2].

During oocyte maturation, many of the synthesized proteins are stored and become active after fertilization. After ovarian hyperstimulation, 43 unique proteins were identified in FF responsible for inflammation and regulation of acute-phase, complement and coagulation, response to wounding, protein-lipid complex/lipid metabolism and transport, and cytoskeleton organization. Core protein alpha-1-antitrypsin was differentially expressed in the FF and sera of younger and older women [31]. Similarly, the differential expression of transferrin, complement component C3, haptoglobin, and alpha-1-antitrypsin were able to differentiate the women subjected to controlled ovarian hyperstimulation from natural cycles [32]. A panel of 11 potential FF protein biomarkers (haptoglobin alpha, mitochondrial integrity genome (ATPase), apolipoprotein H, dihydrolipoyl dehydrogenase, lysozyme C, fibrinogen alpha-chain, immunoglobulin heavy chain V-III (increased), antithrombin, vitamin D-binding protein, and complement 3) were used to differentiate the successful and unsuccessful IVF outcomes [33].

43.5 Protein Biomarkers and Embryo Quality

Embryonic regulatory proteins play a vital role beginning from fertilization to embryo implantation. Differential protein expression was observed between the different stages of blastocyst development. Proteins involved in the apoptotic and signal inhibition mechanism were prominent in blastocysts. Ubiquitin is considered as a potential biomarker for monitoring blastocyst development [34, 35]. Similarly, Tedeschi et al. reported that DEPs involved in the ubiquitin system (LAMC1, EEF1A2, RANGAP1, UCHL1, BEND4, PCGF1, UBQLN2, UBC, and PSMD4) that are present in the blastocoel fluid determines the quality of the developing blastocyst [36]. Other proteins such as GAPDH and H2A present in the blastocelic fluid were also associated with embryo implantation [37].

43.6 Secretome Proteomics and Embryo Quality

Embryo-secreted proteins in in vitro culture medium are a good source for noninvasive assessment of their quality. Cortezzi et al. analyzed proteins in the spent culture media and proposed the Jumonji (JARID2) protein as a positive

biomarker for embryo implantation, whereas TSGA10 could serve as a negative biomarker of embryo implantation [38]. During adverse conditions, overexpression of lipocalin-1, a stress protein, leads to the failure of embryo implantation [39]. APOA1 in the spent culture media is associated with better morphology of the blastocysts and embryo implantation [40, 41]. Presence of the human chorionic gonadotrophin (HCGh) isoform in the spent culture media serves as a negative biomarker for embryo development [42].

43.7 Endometrium Proteomics

Besides the selection of high-quality embryo, a well-thickened endometrium is another factor that determines implantation and subsequently the establishment of pregnancy. Protein-protein interactions between the embryo and endometrium also have an impact on embryo development and implantation. In-depth proteomic knowledge about the endometrium and its secretions will help decipher the role of the vital proteins involved in endometrial receptivity toward embryo implantation [43]. Inflammation of the endometrium is associated with fertility-related problems. Reception of embryo by the endometrium is essential for implantation and development of the embryo. In ART, unsuccessful pregnancy is mainly due to poor endometrial receptivity even though the superior-quality embryo is involved in ETs. In general, most of the endometrial proteins are differentially expressed during the proliferative and secretory phases [44–46]. Glutamate NMDA receptor subunit zeta 1 precursor and FRAT1 are two examples of such DEPs [44].

Differential expression of proteins in the endometrial secretome affects the endometrial receptivity of fertile and infertile women [47]. High-throughput screening of proteins in receptive versus non-receptive endometrium identified annexin A2 and stathmin as potential biomarkers of endometrial receptivity [19]. In patients undergoing IVF cycles with administration of progesterone, the progesterone receptor membrane component 1 (PGRMC1) and annexin A6 (ANXA6) proteins are involved in acquiring endometrial receptivity [20], whereas the ACO2, CDC5L, GNAS, ARF1, ANPEP, SERPIND1, and SEC23B proteins were found to be differentially expressed in GnRH antagonist-based ovarian stimulation protocols [48].

43.8 Cumulus Cell Proteomics

The oocyte is surrounded by layers of cumulus cells (CCs) and its maturation is dependent on communication with the CCs. Oocyte grading and its fertilization capacity is assessed by the CCs morphology [49]. Human CCs serve as biomarkers for embryo and pregnancy outcomes [50]. However, studies on the proteomic profiling of CCs are very limited. Hamamah et al. demonstrated the difference in the protein expression profiles of human CCs with different ovarian stimulation protocols. A correlation between the protein profile of CCs and the maturation and fecundity of the oocytes was established [51]. In patients undergoing ICSI, 72 CC proteins were differentially expressed in pregnant and non-pregnant women. Most of these proteins were binding proteins and enzymes. CCs proteomic profiling was able to identify potential biomarkers for blastocyst formation and successful pregnancy [52].

43.9 Metabolomics and Embryo

The profiling or fingerprinting of the end products (metabolite) of metabolism using a nontargeted approach [53] is known as metabolomics. The metabolome refers to the complete set of small molecules (<1 kDa) such as hormones, signaling molecules (amino acids, lipids, and nucleotides), and secondary metabolites. The concentration and composition of the metabolome reflect the output (end product) of gene expression at a metabolomics level, i.e., they act as an interface between the cellular genotype and phenotype. In general, the metabolites produced are lesser than the mRNA transcripts and proteins synthesized by the cells. Therefore, metabolomic analysis is comparatively faster than other omics (genomic or proteomic) analysis. Not only does alteration in protein expression affect cellular processes, it also disturbs the dynamic balance of the metabolites leading to a pathophysiological state [18]. Metabolomics deals with a different diverse class of molecules such as amino acids, carbohydrates, fatty acids, and carboxylic acids. Biofluids that perfuse the cells are considered as the ideal suitable material for the analysis of the metabolites [54].

Metabolomics could potentially be more informative than genomics, transcriptomics, or proteomics because the metabolites represent the final products of cellular regulatory processes and may signify the final response of a biological system to genetic factors and/or changes in its environment [55] (Fig. 43.2). Targeted and nontargeted approaches have been used to study the metabolomics. Targeted metabolomics have been practiced for the past 30 years and known metabolites are quantified noninvasively, which mainly include glucose and amino acids. However, the introduction of high-throughput platforms such as Raman spectroscopy and ^{1}H nuclear magnetic resonance (NMR) spectroscopy, near infrared (NIR) spectroscopy, electrospray ionization mass spectrometry (ESI-MS) [56], and direct injection mass spectrometry (DI-MS) [57] provided complete profiles of the metabolites with minimal amount of sample.

Metabolomics has been previously used to demonstrate metabolites in a cellular system under a given set of physiological conditions and deal with a diverse class of molecules with different properties. Nowadays, it is used to determine the growth of embryo and a favorable environment under in vitro conditions. It also assesses the connections between substrates and pathways, as well as interactions between metabolic reactions within the embryo. The main objective of metabolomic profiling is to identify and quantify all the metabolites in a biological fluid (FF, endometrial secretion,

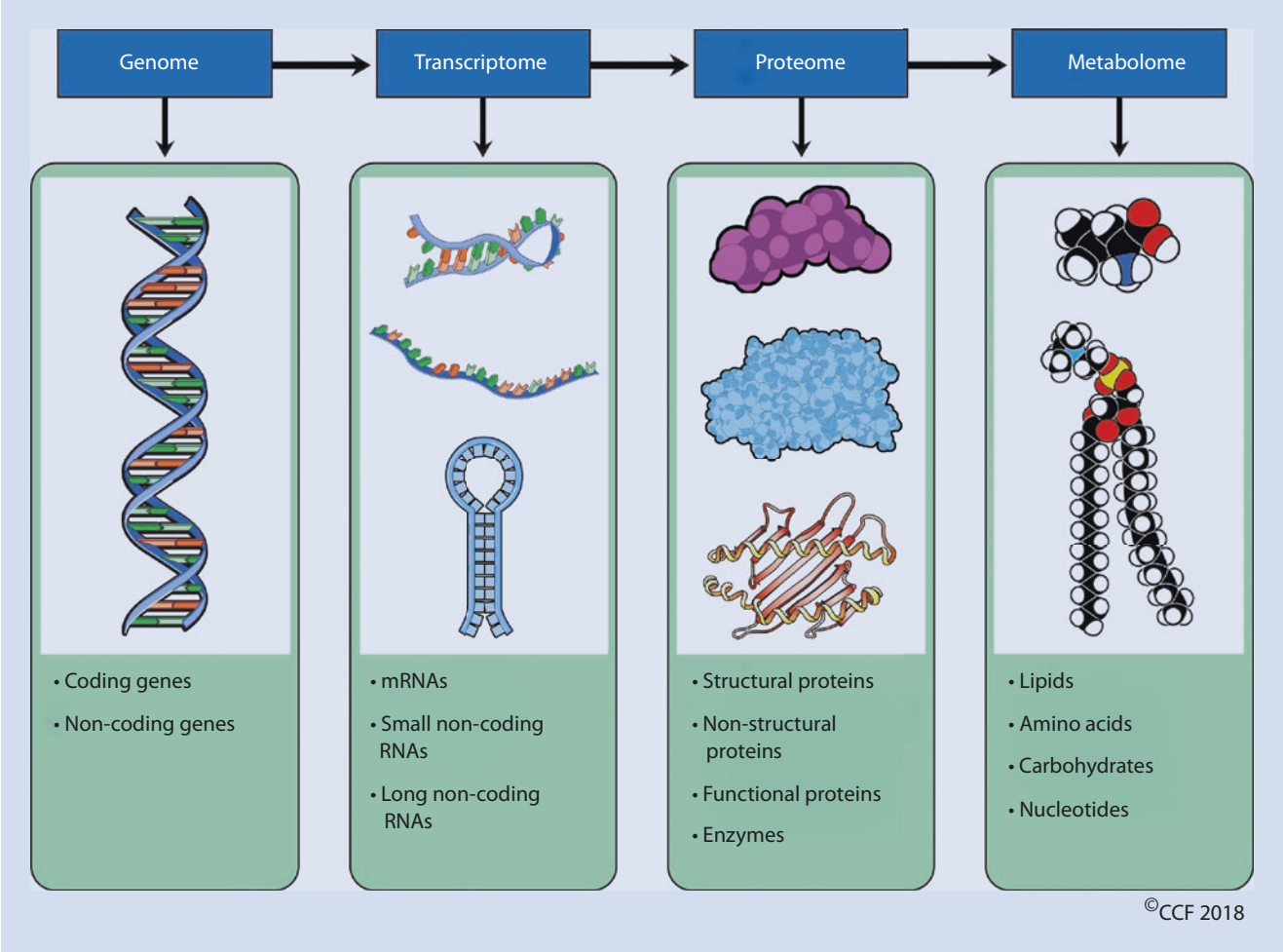

Fig. 43.2 Interaction of the genome, transcriptome, proteome, and metabolome with the environment manifests as a biological phenotype. Metabolites are thus the final product of interactions between the gene and the environment

and spent embryo culture medium). Metabolites are measured in embryo culture medium via noninvasive methods to avoid damage to the developing embryo. Even though metabolomic profiling does not correlate with the fertility outcome, it provides complete information and metabolite composition in the developing oocytes and embryos [58]. This has potential implications in assessing the quality of the embryo.

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43.9.1 Commonly Used Metabolomics Techniques

New techniques were applied to study the complete array of small molecules in embryo metabolism [59, 60]. Several platforms such as MALDI, MS-coupled with gas or liquid chromatography (GC or LC), NMR, NIR, or Raman spectroscopy have been used to assess the metabolomic activity of embryos [17, 18, 61]. Prior to the processing of samples for metabolomic studies, metabolic activity in biofluids is halted by storing the samples at −80 °C or by snap freezing in liquid nitrogen. The information generated through these high-throughput techniques are complex and require powerful statistical and bioinformatics tools for analysis and interpretation.

43.9.2 GC-MS and HPLC Coupled with MS

MS is a powerful analytical technique for rapid and quick quantification of metabolites with high sensitivity in samples. MS-based metabolomic analysis is well-suited for samples with a complex mixture of compounds and even to detect low-abundance metabolites [62]. MS alone cannot resolve all the compounds; it requires comprehensive sample preparation steps. When combined with advanced GC or HPLC separation techniques, the analytes are detected more efficiently by the instruments used [63]. The abundance and expression of the metabolites are quantified by spectral deconvolution. Capillary electrophoresis MS is applicable to samples with very low volumes to analyze both polar and thermolabile compounds. However, GC-MS platform is preferred due to the poor reproducibility of capillary electrophoresis MS [64].

43.9.3 NMR Spectroscopy

Nicholson et al. used the NMR technique for the very first time to profile the metabolome in biofluids [53, 65]. Later, the technique was widely used to profile the metabolome of different biological samples. The NMR technique can analyze the metabolites in both solid and fluid samples directly without sample processing. However, this technique requires larger volumes of samples for analysis and is less sensitive than MS particularly to low-abundance metabolites. In the field of ART, high-resolution nuclear magnetic resonance (^{1}H-NMR) spectroscopy has been successfully employed to identify specific metabolic changes in follicular fluid and spent embryo culture medium [66–68]. Apart from the complete profiling, NMR is able to detect isotopes such as ^{13}C, ^{31}P, and ^{15}N [69]. Therefore, metabolomics can be used in the future to decipher the molecular pathways regulating embryo development.

43.9.4 Raman and Near Infrared Spectroscopy (NIR)

There are noninvasive techniques with a rapid turnaround time (<1 min/sample) that do not require any preparatory step or reagents for sample analysis. They are based on the principle that chemical bonds absorb light energy when samples are subjected to light or electromagnetic radiation. This results in the stretching of the bonds, which is compared with the functional groups or single bonds of the molecules to identify the unknown metabolites. Raman spectroscopy measures the vibration of bonds within the functional groups present within the sample [70], whereas NIR spectroscopy measures the intensity of overtones and combination vibrations of the bonds [61]. These two spectroscopy techniques are frequently used in several metabolomic studies, including assessing the metabolomic profile of human embryo spent culture media [60, 71–73]. Viability index for the embryos has been developed using a mathematical model-based algorithm in association with the metabolomic profile [60].

43.9.5 The ViaMetrics-E™ Procedure

ViaMetrics-E™ is a highly sensitive and rapid method performed on-site to assess embryo quality based on the metabolomic profile of the spent culture medium. Initially, morphologically selected embryos from the same patient are cultured individually. A small volume of spent culture medium is collected and loaded into sample cells using a gel loading pipette. The sample cell is then inserted into the ViaMetrics™ instrument, which scans the same sample thrice. It then measures the metabolites present in the culture medium using NIR spectroscopy. Depending on the composition of the metabolites in the spent culture medium, the viability score of the embryo is assessed. Embryos with higher viability scores have higher reproductive potential [74]. Further, the embryos are ranked based on the viability score obtained from a single patient and are selected for the single embryo transfer (SET) technique [58].

This procedure can provide the features of the embryo, but cannot improve the quality of poorly graded embryos. It is a personalized test and the quality of embryos can only be compared among the patients. Due to the inconsistency and inaccuracy in the measurement, this technique is not used in the clinical laboratory.

43.9.6 Bioinformatics Analysis of Metabolomic Data

Complexity in the bioinformatics analysis of metabolomic data depends on the metabolomics approach used to identify metabolites. Analysis of targeted metabolic profiling data is simpler than the data generated from the nontargeted metabolomics approach, because of targeting the specific compounds in the sample. Data normalization is done prior to the bioinformatics analysis of the metabolomic data to overcome the variation in the expression of the metabolites already present in the culture media or spent media. In silico analysis provides the functional role of all the metabolites and their involvement in different molecular pathways [75].

Efficacy of the metabolomics technique to assess embryo viability is verified based on predictive models. Predictive models are developed by applying the mathematical background (decision trees, artificial neural networks, genetic algorithms, etc.) to the metabolomics data. The power of the model is determined by receiver operating characteristics (ROC) curves analysis, to evaluate the sensitivity and specificity of the predictive model [76] (Fig. 43.1).

43.10 Metabolome of Follicular Fluid

FF is considered as a potential source of metabolic biomarkers in determining oocyte quality, as they reflect the physiological status of the oocyte. Metabolomics profiling of FF provides a snapshot of all the metabolites based on the sensitivity of the technique. Piñero-Sagredo et al. (2010) reported 42 different metabolites in the FF. Metabolites such as glucose, lactate, acetate, acetoacetate, pyruvate, and beta-hydroxybutyrate present in the FF were associated with anaerobic glycolytic metabolism in follicles and fatty acids synthesis having influence on the fertilization process [77]. The composition of palmitic acid, arachidonic acid, and stearic acid was significantly different between the cleaved and non-cleaved embryos among the 21 identified fatty acids in FF [68]. Similarly, other metabolites such as lactate, phosphocholine, glucose, and high-density lipoproteins (HDL) have been demonstrated as potential biomarkers to determine oocyte quality for development into a two-cell embryo [71].

In repeated IVF failure patients (RIF), increased levels of amino acids and decreased levels of dicarboxylic acids, cholesterol, and organic acids can serve as biomarkers for predicting the fertilization outcome in RIF conditions [78].

43.11 Metabolic Biomarkers of Embryo Culture Media

Metabolic turnover is crucial for a preimplanting embryo to grow and achieve successful pregnancy. Accordingly, the nutrients and metabolites secreted by the embryos in the culture media have been studied as potential predictors of embryo quality using noninvasive tests. Raman spectroscopy has detected an increased amount of –SH group metabolites and decreased amount of –CH and –NH metabolites in the culture of embryos with high implantation potential [60]. High levels of glutamate were present in the secretome of the embryos resulting in positive pregnancy [79]. Similarly, in another study, NMR-based metabolic profile of spent embryo culture medium showed formate:glycine and citrate:alanine as biochemical markers for the selection of viable embryos [72]. In addition, alanine and leucine levels can be used as noninvasive markers of embryo quality in SET technique [80].

Bellver et al. (2015) reported different metabolic profiles in obese women and 551 metabolites differentially regulated in the culture medium of day 3 embryos. Saturated fatty acid composition was low in the secretome, affecting embryo quality [81]. A metabolic profiling approach was also used to detect aneuploidy in IVF embryos. Differentially expressed metabolites androsterone sulfate and caproate and the isoleucine spectral signals can serve as potential noninvasive biomarkers to detect Down's syndrome prior to embryo transfer [82].

43.12 Metabolomics of Endometrial/ Blastocoele Fluids

It is very important to understand the dynamic cellular processes and metabolism of the embryo and endometrial receptivity, as they are directly associated with embryo implantation or pregnancy failure. Metabolites such as ATP, glucose-6-phosphate, lactate, 6-phosphogluconic acid, glutamic acid, and α-ketoglutarate present in the blastocoele fluid are vital for embryo development [83]. MS-based analysis can also detect most of the metabolites of potential interest responsible for assessing embryo quality in the blastocyst [84].

Metabolites, especially the lipid composition of endometrium, decide endometrial receptivity toward embryo implantation. Lipidome analysis describes prostaglandins E_2 and $F_{2\alpha}$, responsible for blastocyst development during implantation, as potential biomarkers to predict endometrial receptivity [85].

43.13 Metabolic Assessment of Oocyte and Embryo Quality

Oocyte and embryo quality assessment is one of the major objectives of embryologists to increase pregnancy rates during human IVF. The assessment must be rapid and reliable and evaluated based on the embryo viability score. In general, embryos with high viability score are preferred in the ET technique. Metabolic profiles predict embryo quality and viability at the early stages of development (day 3 and day 5 embryos) [86]. Advanced spectroscopy techniques such as Raman determine embryo viability with a sensitivity of 86% and a specificity of 76.5%, whereas NIR has a sensitivity of 75% and a specificity of 83.3% [60]. Nonetheless, these techniques are unable to prove their advantage over conventional morphology grading of embryo in terms of pregnancy and live birth rates [87, 88]. It was the same with embryos selected using NMR-derived metabolomics, as these did not exert significant differences on pregnancy outcomes compared to embryo morphology assessment [67, 89]. Currently, there is no evidence that metabolite profiling can improve fertility outcomes. However, it provides the nutritional and developmental status of the oocyte and embryo. In the future, it has a pronounced potential diagnostic value to identify the etiology for pregnancy failure.

43.14 Embryo Selection

Appropriate selection of good-quality embryos is compulsory to achieve successful IVF results. In routine IVF practice, to improve the success rates, multiple ETs were done following the superovulation protocol. This may result in multiple gestations complicating the pregnancy, including preterm delivery, low birth weight, and dramatic increase in the relative risk for cerebral palsy [4, 5]. Therefore, the SET technique has been recommended to minimize the risk of multiple gestations [58, 74].

Since a decade ago, morphological assessment of embryos was practiced to select the superior quality embryo. However, it is highly subjective and fails to identify genetic or epigenetic defects [61]. However, metabolomics has a great potential as a noninvasive method to replace the existing techniques for embryo selection for SET. To be more effective, the technique has to measure embryo viability with high accuracy but without causing any damage to the embryo.

43.15 Proteomic and Metabolomic Biomarkers

In the past 20 years, various molecular biomarkers have been proposed for embryo evaluation in the field of ART. Sophisticated metabolomic and proteomic techniques proved to be promising platforms for discovering these biomarkers to assess embryo quality. Bioinformatics analysis of

Table 43.1 Recent proteomic studies related to embryo viability assessment

Study	Technique used	Sample	Key findings
Oh et al. [90]	TMT isobaric labeling and LC-MS	FF from POR patients	Inflammatory regulating proteins are differently expressed. PZP, RENI, SRPX, and SHBG are validated as biomarkers of POR
Bianchi et al. [2]	Computational and manual data mining	FF	Metalloproteinases, thrombin, and vitamin-D receptor/retinoid-X-receptor-alpha are suggested biomarkers of oocyte quality
Tedeschi et al. [36]	nLC-ESI- MS/MS	Blastocoel fluid from women aged <37 years and ≥37 years	DEP related to ubiquitin system affects blastocyst survival and implantation
Dominguez et al. [91]	Affymetrix Procarta immunoassay	Spent media of implanted and non-implanted embryos	IL-6 was overexpressed in the culture medium of implanted embryo
Wu et al. [32]	MALDI-TOF-MS	FF from infertile women undergoing COH and natural ovulatory cycles	DEPs were involved in the regulation of immune and inflammatory responses. Transferrin, complement component C3 (C3), haptoglobin, and alpha-1-antitrypsin (AAT) are validated biomarkers
Regiani et al. [92]	Tandem MS	FF from endometriosis and endometrioma patients	Coagulation process and sterol metabolism pathway were enriched in endometriosis, whereas response to reactive oxygen species, oxygen transport, and hemoglobin complex were enriched in endometrioma
Garrido-Gomez et al. [20]	DIGE, MALDI-MS	Endometrial tissue from receptive and non-receptive patients	Significant difference was observed in carbohydrate biosynthetic process and "nuclear mRNA splicing" pathways. ANXA6 and PGRMC1 are predictive biomarkers
Bianchi et al. [31]	2D-gel electrophoresis and MALDI-TOF/TOF	FF from COH women	Reported alpha-1-antitrypsin as biomarker for COH under IVF procedure
Nyalwidhe et al. [41]	LC-ESI-MS-MS	Culture medium of 2–3-day-old embryos	Apolipoprotein A-1 predicts the embryos with high and low implantation rates

2D two-dimensional, *COH* controlled ovarian hyperstimulation, *DEP* differentially expressed protein, *DIGE* difference gel electrophoresis, *ESI* electron spray ionization, *FF* follicular fluid, *LC* liquid chromatography, *MALDI* matrix-assisted laser desorption/ionization, *MS* mass spectrometry, *POR* poor ovarian response, *TMT* tandem mass tag, *TOF* time-of-flight

the massive proteomic and metabolomic data using advanced software tools provides first-hand functional information about the proteins and metabolites regulating biological pathways related to oocyte and embryo development. Some of the potential biomarkers used for the prediction of embryo quality are listed in Tables 43.1 and 43.2.

43.16 Future Directions

ART specialists are in great need of an accurate and precise technique to select the highest-quality embryo for ET to increase the success rate of pregnancy. In addition to recent advances in the proteomic (LC-MS/MS, MALDI-TOF) and metabolomic (Raman, ^{1}H NMR, NIR, ESI-MS) techniques, extensive research is required to validate biomolecules in a large population. The clinical acceptability of these techniques depends on increasing the number of available studies and powerful meta-analysis that can recommend the suitability of biomarkers in embryo evaluation/selection.

Another forthcoming area of interest would probably be to reduce multiple ET by promoting SET procedure with the embryo that results in successful pregnancy. This mainly relies on the proteomic and metabolomic techniques for selecting the embryos. Publications on metabolomic and proteomic profiling are growing exponentially, showing a promise for its use in embryo selection techniques. Integrative analysis of data from both the omics may interlink and define the pathways involved during embryo cleavage and implantation. Further automation of these high-throughput techniques with user-friendly software as well as sample handling that allows multiple sample analysis at a single time point can increase the efficiency in embryo selection. Ultimately, it is hoped that patients will benefit from the application of advanced oocyte and embryo selection methods, with better possible ART outcomes.

Table 43.2 Recent metabolomic profiling studies that have an impact on embryo development and assessment

Study	Technology used	Sample	Key pathways and metabolites
Zhang et al. [66]	^{1}H-NMR	Follicular fluid	In PCOS: glycoprotein, acetate, and cholesterol are upregulated, but lactic acid, glutamine, pyruvate, and alanine are downregulated
Wallace et al. [72]	^{1}H-NMR	Spent embryo culture media	Increase in formate to glycine ratio and decrease in citrate to alanine ratio are indicators of positive pregnancy
Xia et al. [78]	GC-MS	Follicular fluid	Increased expression of valine, threonine, isoleucine, cysteine, serine, proline, alanine, phenylalanine, lysine, methionine, and ornithine and decreased expression levels of dicarboxylic acids, cholesterol, and some organic acids were observed in RIF patients
Vilella et al. [85]	LC-MS/MS	Endometrial fluid	Endometrial receptivity markers: PGE_2, and $PGF_{2\alpha}$
Zhao et al. [73]	Raman	Spent embryo culture medium	Sodium pyruvate and phenylalanine are important for embryo development
Wallace et al. [71]	^{1}H-NMR	Follicular fluid	In non-cleaved oocytes, glucose and HDL levels were elevated, whereas lactate, choline, phosphocholine, and glycerophosphocholine were reduced
Sánchez-Ribas et al. [82]	LC/GC-MS and NMR	Spent embryo culture media	Androsterone sulfate and caproate are potential biomarkers of Down's syndrome
Bulletti et al. [83]	HPLC-MS	Blastocoele fluid	ATP, glucose-6-phosphate, lactate, 6-phosphogluconic acid, glutamic acid, and α-ketoglutarate are vital for embryo development

GC gas chromatography, *HDL* high density lipoprotein, *^{1}H-NMR* proton nuclear magnetic resonance spectroscopy, *HPLC* high-performance liquid chromatography, *LC* liquid chromatography, *MS* mass spectrometry, *PCOS* polycystic ovary syndrome, *RIF* repeated IVF failure

43.17 Conclusion

The conventional system of morphological grading and evaluation of embryo requires highly trained and skilled personnel. Proteomics and metabolomics are two complementary omics platforms that may be used for embryo selection in the field of ART. Combining the proteomic and metabolomic profile of the embryo can provide the complete molecular status of the secretome. Moreover, a plausible explanation for altered molecular composition and validation of biomarkers in a large cohort population might further support the acceptance of these new technologies in the clinical setting. Future investigations focusing on the design of new devices by integrating both the omic platforms to assess embryo viability could potentially make a significant contribution toward the ART field. When proven as an efficient technique, the omics may possibly help increase live birth rates using the SET technique, to provide better-quality service for infertile patients.

Review Questions

1. Are the noninvasive techniques used in the assessment of embryo quality beneficial in the IVF clinic?
   - Yes, these noninvasive techniques would help avoid any damage to the embryo during the embryo assessment leading to embryo selection.
2. Why is there a need for molecular biomarkers (metabolomic and proteomic) to assess embryo quality and viability?
   - Molecular biomarkers are able to precisely point out the biological processes and the nutritional status of the embryo. This would help in the selection of a high-quality embryo.
3. Is spent embryo culture medium a good source to assess embryo viability?
   - Yes, because the spent culture medium is the secretome of the embryo. It acts a good source of diagnostic material for embryo evaluation and selection in IVF practice.
4. Could the merging of the two omics (proteomics and metabolomics) platforms lead to better embryo selection?
   - These high-throughput platforms are able to generate enormous amounts of information

which are different for each omic study (proteomic and metabolomic). Combining two data sets will provide the link and connection between both processes and subsequently help in better assessment of embryo quality.

5. What is the impact of metabolomic and proteomic tools on the single embryo transfer technique?

Proteomic and metabolomic profiling will help ensure that the superior-quality single embryo is selected to achieve pregnancy. Single embryo transfer avoids multiple pregnancies, which is considered as a significant health issue in pregnant women.

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Amino Acid Turnover as a Biomarker of Embryo Viability

Christine Leary, Danielle G. Smith, Henry J. Leese, and Roger G. Sturmey

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Learning Objectives

- To define the benefits of embryo selection and/or ranking during IVF treatment
- To review how traditional embryo assessment may not be sufficient for efficient embryo selection and what alternative options exist
- To describe how amino acid turnover is related to embryo development and viability
- To review the technological aspects of performing amino acid measurement from spent culture medium
- To present how amino acid profiling may be used as an additional tool for embryo selection

Most of the rise in multiple births observed since the 1980s has been attributed to the increased use of IVF and the practice of replacing more than one embryo into the uterus in order to improve the chances of success. In response to the unacceptably high risks associated with multiple births, several countries have introduced single embryo transfer policies. However, to maintain acceptable pregnancy rates, the pressure to identify the most viable embryo for transfer has intensified.

The most widely used method of embryo selection has been to assign scores on day 2 or day 3 post insemination on the basis of the embryo's cleavage rate and morphological appearance. Studies have shown that correlations exist between these observational cues of embryo quality and their capacity for continued development and subsequent implantation. However, appropriate temporal developmental patterns are critical to predicting embryo viability. For example, Cummins et al. [1] assigned scores based on the ratio between the time at which an embryo was observed at a given stage of development and the expected time taken to reach that particular stage and showed that pregnancy rates were lower for those with above or below average scores. In a powerful study based on observations from single embryo transfer, Giorgetti et al. [2] demonstrated the additional benefit of including a morphology score. Morphological markers linked to implantation include blastomere size and regularity [3] and the presence of fragmentation [2], multinucleation [4], granulation, and zona thickness. Fragmentation is the most commonly reported observation, and >20% fragmentation is regarded by many as biologically deleterious. The caveat is that morphological assessment is highly subjective, and there is evidence that the pattern of fragmentation may be more important than fragmentation per se [5].

Blastocyst formation occurs between day 5 and day 7 and is a critical developmental milestone. Approximately 50% of human zygotes have the potential to reach this stage [6]. Aneuploidy is commonly associated with developmental arrest; hence, the proportion of embryos with chromosomal abnormalities is lower in blastocysts compared to cleavage-stage embryos. The method of grading blastocysts is similar to that of grading cleavage-stage embryos; blastocysts are scored on how similar they are to the *morphological ideal*. This involves grading according to the presence of a cavity, a visible inner cell mass (ICM), regular trophoblast (TE) cells, degree of expansion, and zona thinning. The ICM and TE are graded according to how tightly cells are packed and the number of cells, particularly in the ICM.

Selection of the most viable embryo to transfer and at which stage of development remains one of the most challenging aspects of in vitro practices. There is little consensus regarding the observations to make and how frequently to record them. Many schemes use a combination of criteria, including the addition of pronuclear morphological scores, early cleavage, and more controversially aneuploidy screening, as adjuncts to those used routinely as described in this section.

Attempts to draw conclusions on the effectiveness of current observational embryo grading and selection tools have been hampered by a lack of generic terminology and methodology. There is a clear need for a standard embryo scoring/selection system, and attempts to introduce external quality assessment schemes are currently being piloted in the UK. It is hoped that with the introduction of consistent terminology and reduced operator scoring variability, this will permit large-center studies and allow more definitive correlations to be drawn, leading us closer to defining what indicates a viable embryo.

44.1 Why the Need for Objective Markers of Viability?

Embryo selection is currently based on operator observations of embryo developmental and morphological score on the assumption that these two features are affected by intrinsic factors and as such are a reflection of genetic viability of an embryo. They may also be influenced by extrinsic factors such as the culture environment. For example, Lane et al. [7] showed that media composition can affect cleavage rates, and improvements to embryo culture systems have supported the increased development rate of embryos to the blastocyst stage such that extended culture and blastocyst formation is now frequently used to select competent embryos for transfer.

Blastocyst transfer is not a suitable strategy for all patients as it may result in fewer embryos being available for freezing and an increased risk of canceled cycles. Furthermore, embryos are able to adapt to their environment, and if the in vitro environment is suboptimal, prolonged culture may reduce embryo viability or have other more subtle effects such as an undesired modification of epigenetic changes or metabolic dysregulation, although further rigorous evaluation is required. In other words, there is a need to find the safest and most effective method to help patients achieve a single healthy pregnancy. An accurate system for assessment of embryo viability will enable practitioners to counsel patients on their chances of conception and reduce the numbers of embryos transferred.

44.2 Amino Acid Profiling

The search for biomarkers of embryo viability in spent embryo culture medium has a long history. Potential markers have included the consumption of glucose, lactate, pyruvate, oxygen, and glutamine and the appearance of proteins such as sHLAG and PIF. However, there is increasing evidence to support the proposition that *amino acid profiling* reflects the developmental capacity of early embryos. By measuring a group of 18 compounds, amino acid profiling provides a snapshot of embryo phenotype by virtue of the many roles played by amino acids during embryo development, some of which are considered below. Thus, amino acid profiling differs from conventional metabolic assays, where typically, only one or two metabolites are measured.

Since the first application of amino acid profiling of mouse embryos was published in 1994 by Lamb et al. [8], the technique has been applied to bovine [9–12], porcine [13], and, critically, human embryos [14–16]. Without exception, in those studies in which it was addressed, the research has demonstrated that the amino acid composition of spent culture medium, i.e., amino acid profile (AAP) of *viable* embryos differs from that given by subviable embryos. These studies have used different indicators of embryo quality, from blastocyst formation [14], through molecular scale observations of DNA damage [15], to correlation with clinical pregnancy [16]. Crucially, the link between AAP and viability is independent of embryo morphological grade and is apparent from day 1 to 2 or day 2 to 3 post insemination.

44.3 The Biology of Amino Acids

It is well recognized that amino acids have a variety of roles in the cell in addition to their traditional function as constituents of proteins. Some such roles are listed below with a brief consideration of their contribution in the early embryo.

44.3.1 Protein Synthesis

Protein synthesis is relatively low during the cleavage stages of preimplantation development but increases sharply with blastocyst formation [17, 18]. This is consistent with the observation that the protein content of preimplantation embryos is relatively stable during cleavage before increasing in the blastocyst, with the onset of true growth [19]. This is reflected in the uptake of certain amino acids from within a mixture when provided in culture, as seen in bovine [9], porcine [20], and human [14] embryos.

44.3.2 Energy Sources

Approximately 14% of human energy needs are provided by the oxidation of protein, ultimately provided by the diet, and it is likely that early embryos cultured with a physiological mixture of amino acids will exhibit a similar figure. When cells are provided with an excess of protein or amino acids, then once the requirement for the synthesis of protein and other amino acid-derived products has been met, they will first tend to be oxidized, in preference to carbohydrate and fat, since mammalian cells have no capacity to store amino acids or protein. In most mammalian cells, protein synthesis is one of the two major consumers of energy, the other being the Na+, K+, and ATPase; their contributions to the energy budget is ~25% each, though with considerable variation depending on the cell type and physiological state [21–23].

This issue was addressed for mouse blastocysts by Leese et al. [24], who reported considerable variation in the energy cost of protein synthesis depending on the methodology and assumptions. Despite these caveats, it is most likely that protein synthesis will be a major component of energy homeostasis and, in the context of the present discussion, provide a good marker for the overall nutritional requirements of the early embryo.

44.3.3 Nucleotide Synthesis

Glutamine, aspartic acid, and glycine provide carbon and nitrogen atoms for purine and pyrimidine de novo synthesis—metabolic pathways which are essential to mouse preimplantation development [25]. A key amino acid is thought to be glutamine, which is readily consumed by rapidly dividing cells, and in early embryos [19], in addition to providing purine and pyrimidine nucleotide precursors, it acts as an energy source.

44.3.4 Osmolytes

Notable among amino acids which act as osmolytes during mouse preimplantation development are glycine, betaine, glutamine, and proline [26].

44.3.5 Provision of 1-Carbon Units

Methylation reactions, which are integral to appropriate gene silencing by methylation pathways, require functioning methyl group cycling, in which the amino acid methionine is a key component together with derivatives of the vitamin folic acid.

44.3.6 Signaling Molecule Precursors

In the context of the early embryo, perhaps, the best-characterized example of the function of amino acids as precursors of signal transducers is the formation of nitric oxide from arginine. The role of NO has been the subject of a number of studies in the early embryo. For example, Manser et al. [27] reported data consistent with NO having an obligatory

role in mouse preimplantation development, in terms of Ca^{2+} signaling in mitochondria [28], and Lipari et al. [29] reported an association between human embryo-mediated NO production and subsequent blastocyst formation.

Thus, the biological rationale for the ability of amino acids to act as biomarkers of early embryo viability is compelling. The practicalities of assaying amino acid metabolism by early embryos are now considered.

44.4 How to Measure Amino Acids

44.4.1 Principle of Chromatography/High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is a form of column chromatography, in which compounds of interest are separated according to their chemical and physical properties. A sample containing a mixture of compounds of interest, in the present context, amino acids, is diluted into a liquid carrier, the *mobile phase*, and pumped under high pressure onto a *solid-phase* column. The column impedes the passage of the amino acids as they interact with the packing substrate within the column. By modifying the conditions of the mobile phase, the amino acids are washed off, or *eluted*, from the column and detected by an appropriate system. Each amino acid differs in terms of structure and chemistry and on this basis interacts with the column in a different way such that it is *retained* on the column for a different length of time. Using this concept of *retention time*, each amino acid may be identified as it is eluted sequentially from the column. Each amino acid generates a quantifiable signal, which is directly proportional to the signal given by a standard mixture of amino acids at a known concentration. Using this simple approach, it has been possible to measure changes in the amino acid content of spent embryo culture droplets to a very high degree of sensitivity.

44.4.2 Chemical Properties of Amino Acids

When most of us think of amino acids, we are generally referring to a subset known as the protein amino acids, of which there are 20 naturally occurring L-isomers that are the constituents of proteins. This section will refer to only these naturally occurring amino acids as these are the only relevant subset in the current context. All amino acids share a common structural scaffold, consisting of an amino group, a carboxyl group, and a distinctive side chain denoted as R with each side chain unique to each amino acid. An exception to this general scaffold rule however is proline where the side chain forms a five-membered ring with backbone. Amino acids can be categorized into four main subgroups based on their general chemical nature: weakly acidic, weakly basic, hydrophobic, and polar hydrophilic. These chemical features can be exploited to isolate individual amino acids.

The pI value of most amino acids is approximately six, meaning that at neutral pH, amino acids ionize and are typically zwitterionic. That is, they exhibit both a positive charge on the amine and a negative charge on the carboxylate; however, the pI of the side chains of the amino acids varies, which affects the overall charge of the particle. It is the chemical and structural properties of the side chain that dictate which subgroup an amino acid falls into, affecting charge, reactivity, and hydrogen-bonding capacity of the amino acid. Therefore, changing the pH and ionic strength of the mobile phase can be used to separate amino acids during chromatography, allowing individual amino acids to be isolated. To give one example, under chromatographic conditions of pH 5.9, the solvent, or mobile phase, is positively charged; thus, the first amino acids to elute are *acidic* amino acids: aspartate and glutamate (conjugate bases for aspartic acid and glutamic acid). At this pH, the acidic side chains are deprotonated, meaning they are carrying a negative charge and therefore interact with the positively charged mobile phase rather than the uncharged column substrate and so elute very rapidly. Aspartate has a shorter retention time than glutamate because it has shorter hydrocarbon chain and is therefore slightly less hydrophobic and thus can pass through the column substrate more rapidly than glutamate.

This one example illustrates how on the basis of their chemical and physical properties amino acids may be separated from the mixture present in embryo culture media. It is also important to emphasize that this is an example of *reverse phase* HPLC, distinguished from conventional HPLC because the mobile rather than solid phase in the column is charged (Fig. 44.1). This small distinction is vital as RP-HPLC offers an extra dimension of flexibility with regard to separation parameters since combinations of mobile phases can be run according to the *gradient* method chromatography. Thus, aspartate and glutamate mentioned above can be eluted in a positively charged mobile phase. Conversely, hydrophobic amino acids such as leucine will not elute from a column when the mobile phase is predominantly aqueous and must therefore be eluted in a nonaqueous mobile phase, for example, consisting predominantly of methanol. Gradient HPLC allows the composition of the mobile phase to be changed gradually from a positively charged aqueous buffer, allowing the rapid elution of small acidic amino acids, to a nonaqueous mobile phase, which will enable the elution of hydrophobic amino acids, such as leucine.

44.4.3 Chemical Principles of Amino Acid Derivatization

Amino acids can be detected fluorometrically by derivatizing them with *o*-phthaldialdehyde (OPA). Amino acids need to be derivatized as only a subset is able to detect ultraviolet absorbance (tyrosine, tryptophan at 280 nm). OPA is fluorescent due to the aromatic ring but forms an isoindole upon reaction with amino acids, peptides, and proteins, which can

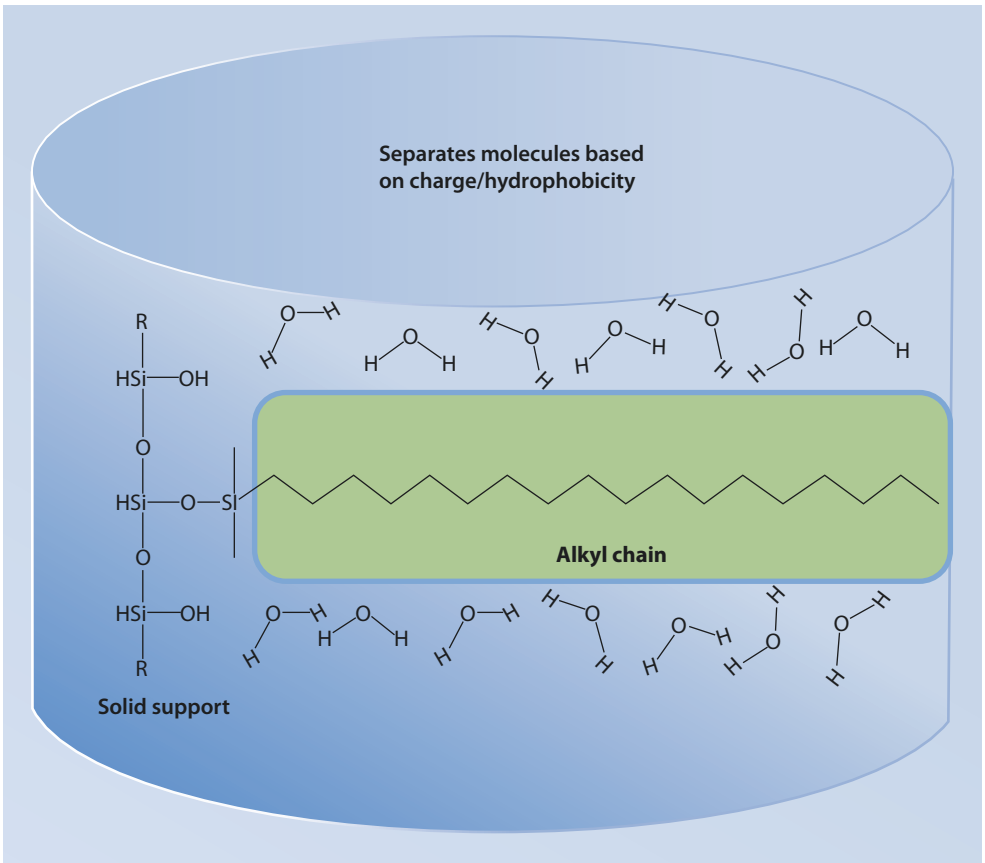

Fig. 44.1 Reverse phase column. The solid support of the column is usually made of chemically inert material of a large pH range, for example, silica. Attached to the inert core are hydrophobic fingers (*green*) made up of alkyl chains. Hydrophilic pockets are formed between the alkyl chains as the column is hydrated with aqueous phase (H_2O) (*blue*). The degree of hydrophilicity of the mobile phase dictates the size of the hydrophilic pockets which in turn affects the ability of the molecules passing through the column to interact with the alkyl chains. These hydrophilic pockets allow charged particles to be retained in the column and separated by manipulating the amount of hydrophilic component in the mobile phase

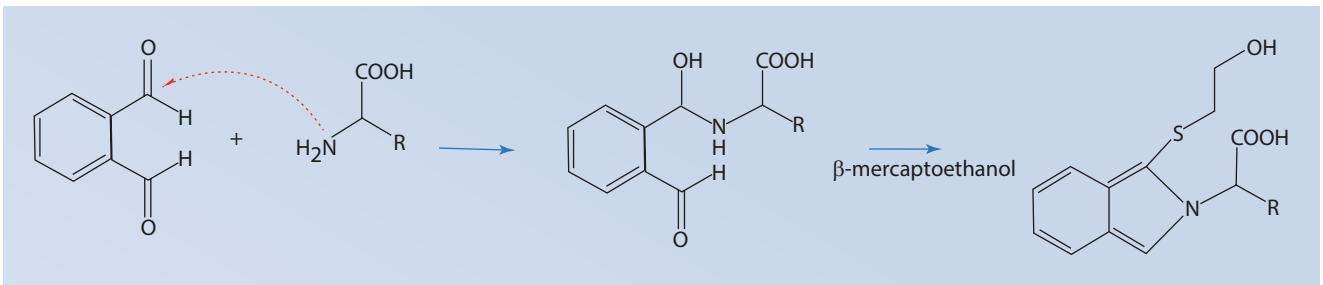

Fig. 44.2 Amino acid derivatization with OPA. The carbonyl of the OPA undergoes nucleophilic attack by the nitrogen of the amine of an amino acid generating intermediate B. In the presence of mercaptoethanol, intermediate B will rapidly convert to the fluorescently detectable isoindole product C

be detected at different wavelengths by exciting at 350 nm and collecting the emission at 430 nm. Derivatization of these moieties with OPA is pH dependent. In order to derivatize amino acids, the pH must be above 9. Under these conditions, OPA reacts with the nitrogen of the backbone amine and in the presence of b-mercaptoethanol forms the isoindole, as shown in Fig. 44.2.

44.4.4 Equipment and Consumables Required

In order to perform HPLC analysis of spent culture medium, an appropriate HPLC system is required. Numerous instrumentation suppliers produce HPLC systems with different options, and most systems are modular. However, regardless of the system used, there are certain features that are important for analysis of amino acids in spent culture medium. The system employed to perform the analysis should have a binary pump system, capable of delivering a changing gradient of elution buffers, and able to pump at a flow rate of up to 2.5 mL min^{-1}. Due to the vast number of samples and controls analyzed, an autosampler, with the capacity to carry out automatic derivatization, is also essential. The autosampler also needs to be temperature controlled and able to maintain the samples at 4 °C to minimize sample degradation during analysis. A column oven is required to ensure that the chemistry on the column is performed at a standard temperature, usually 25 °C. The final component required is the detection system. Since derivatization with OPA yields a fluorescently

labeled amino acid, a fluorescent detector is capable of exciting the eluted samples at around 300 nm and detecting emission at approximately 450 nm.

In addition to the system, selecting an appropriate column is fundamental to efficient separation of amino acids with a mixture. Columns commonly used for this type of separation are silica-based; that is, they are packed with inert silica beads. The mean diameter of the particles used to pack the column is given in the column information. Particle size is important since it affects column separation efficiency; a smaller particle size gives superior separation and smaller interparticle space; that is, the column is *more tightly packed*. However, this will lead to a corresponding increase in the pressure applied to the column, termed the *back pressure*. Prolonged exposure to elevated back pressure (in excess of 250 bar) will significantly impact on the column lifetime; thus, it is necessary to maintain back pressure below 250 bar, preferably below 200 bar. In other words, the choice of particle size is a compromise between separation efficiency and acceptable back pressure. In addition to particle size, the size and volume of the pores on the packing particles must be considered. Pore size is relevant with respect to the size of analytes; separation of small analytes is optimum with smaller pore sizes; a small analyte in a large pore will have more space to fill as it traverses the column; thus, retention times will increase. The pore volume gives an indication of the surface area of the particle; the smaller the pore size and the higher the volume of pores, the higher the overall surface area of the column packing material, which has the effect of increasing column volume and compound retention times.

Next, one needs to consider the physical dimensions of the column; the internal diameter and the length which combined can be used to calculate the overall column volume, known as the bed volume. As a general rule, a smaller column diameter will lead to improved sensitivity and will enable chromatography to be performed at a lower mobile phase flow rate without any effect on linear flow rate of the mobile phase through the column. However, this will lead to a reduction in analyte loading capacity. Similarly, a longer column will improve peak resolution, but at the cost of increased analysis time.

The final key consideration when choosing the column is the carbon load. Typically, for amino acid analysis of spent culture medium, octadecyl silane or *ODS* columns are most commonly used, which are chemically bonded to the silica particles within the column, producing $C_{18}H_{37}$ chains. The length of the carbon chain is an important consideration; as a general rule, the longer the carbon chains, the higher the retention of the analytes. C18 is commonly used in RP-HPLC and is suitable for the separation and detection of amino acids in spent culture medium. This is above the *critical chain length* of ten C molecules [28] and is preferable over an alternative C8 column available from many column manufacturers. The carbon chain length, along with the particle size, number of particles, and pore size (i.e., surface area), influences the efficiency of the column.

It is wise to include a precolumn filter. This is a device through which the samples are passed prior to being loaded onto the column and which retains large contaminants. Precolumn filters are an inexpensive option ensuring column protection from blockages and improving column life-span. Normally, these consist of the same material used in the separating column with a lower packing density.

For the separation of amino acids from spent embryo culture medium, there are a number of key considerations; peak resolution must be maintained, as must repeatability and confidence in repeated assays. A series of five identical standards should be run prior to the loading of any samples to account for interrun variability. The variation between areas given on each of these runs should be less than 5%. However, analysis time needs to be kept to a minimum to ensure sufficient sample throughput. It is therefore necessary to make a suitable compromise, based on the factors described above, to ensure that column properties are suitable for a given application.

It is also necessary to consider the components of the mobile phase. Solvents, such as methanol and tetrahydrofuran (THF), should be *HPLC grade*. These are confirmed as high-purity solvents that have been tested to ensure adherence to strict specifications relating to spectral properties and ionic interactions. Solvents must be stored appropriately but not for prolonged periods as the quality can diminish as the solvents react with the containers in which they are stored. It is therefore preferable to store mobile phases in glass bottles rather than those made of plastic. HPLC-grade water is commercially available; however, it is sufficient to use ultrapure water collected from a suitable water purification system that is capable of producing water with a resistance of 18 Ω. This is preferential to using HPLC water stored for prolonged periods as the impurities arising from the storage container are minimized.

The mobile phase of the HPLC system will contain dissolved gases, and it is important to degas the solvents. This is commonly achieved by the inclusion of an in-line degasser in the HPLC system. The removal of dissolved gases is important to ensure consistent chromatographic performance.

44.5 How to Prepare Samples and Perform High-Performance Liquid Chromatography

Embryos should be placed into small droplets of culture medium containing amino acids; typically, these droplets are 4 μL in volume, placed under oil to minimize evaporation. This is the optimum volume, since it contains amino acids at a concentration low enough to enable detection of small changes but is sufficiently high to ensure that no single nutrient becomes limiting. At the end of the incubation, typically 24 h, the embryo is removed from the droplet, and the droplet is diluted at a ratio of 1:12.5 in HPLC-appropriate water. In practice, 2 μL of sample is added to 23 μL of water in an

HPLC vial. This gives a convenient 25 μL sample volume that is loaded onto the autosampler. At this stage, the samples are derivatized with OPA (see above), using the autosampler device to inject 25 μL of OPA in solution from a reservoir into the sample. After a suitable reaction time, typically 2 min, the sample is injected onto the column commencing separation of the analytes. The eluted peaks are then quantified.

44.5.1 Data Interpretation

The method of amino acid profiling offers significant promise in predicting embryo viability. At present, with conventional scoring methods, even when strict criteria are applied, approximately 25% of embryos graded as top quality will be aneuploid [8], and there is a clear need for improvement. The assignment of embryo *viability scores* based on their amino acid metabolism may provide a superior, more objective, quantitative selection method. Patients may have a cohort of embryos of differing morphology grades and cleavage rates, as well as embryos that exhibit different amino acid profiles indicative of differing embryo viability. The challenge is to discover the biological basis of these differences.

44.5.2 Statistics and Power

The amino acid profile data generated from animal and human models has been related retrospectively to various outcome measures, including blastocyst development and clinical pregnancy. Data from the analysis of 18 amino acids must be interpreted with care. The utilization of individual amino acids is unlikely to be independent of one another; each must be considered as a latent variable where each amino acid will have a unique contribution to the outcome. One must therefore consider how the specific contribution of any given amino acid differs from the remaining amino acids. For this purpose, the technique of principal components analysis is often used to combine the data into a single index that can serve as a measure of embryo viability [16].

Within any dataset, there will be sample variability and measuring errors. The question that needs to be addressed is whether ordinary sampling variance can account for the differences in viability indices between the pregnant and nonpregnant groups. If the possibility of measuring errors is reduced, only then will we be able to ascertain how much of the variability in the outcome can be apportioned to variance in the amino acid data. For instance, if leucine utilization is compared in a pregnant and nonpregnant group, and the variability between the two groups is higher than the within-group variance, we can then say with some confidence that any differences are due to more than sampling variability, and the null hypothesis can be rejected. However, a strength of the system used to quantify amino acid metabolism is the inclusion of an internal reference standard, for example, D-α-amino-butyric acid (DABA), a nontoxic, chemically inert compound incapable of being metabolized by mammalian cell, enabling sampling variance to be corrected.

In significance testing, there are two opposite risks; a type I error is the acceptance of a difference as significant when it is not, i.e., rejecting the null hypothesis when it is true. The converse is a type II error, i.e., accepting the null hypothesis when it is false. The emphasis is generally placed on avoiding type I errors—i.e., false claims. In order to reduce the likelihood of this occurring, emphasis is often placed on significance at 1% level. However, a balance needs to be struck between statistical significance and clinical relevance, and clinically relevant end points need to be defined. The test may aid embryo selection but may not translate to an immediate difference in clinical pregnancy rates. It could, however, improve cumulative pregnancy rates by enhanced selection criteria for cryopreservation. Rigorous follow-up is therefore required to test the merits of amino acid testing.

44.5.3 Relating to Outcome

The question of *cause or effect* poses a challenge, and confounding variables may bias the results. Statistical modeling has been applied to investigate the relationship with known predictors of pregnancy, such as female age and embryo quality, and in the human, amino acid turnover is a powerful, independent predictor of the outcome [16]. However, in order to draw further conclusions from these types of approaches, full randomized prospective clinical trials are now required.

Review Questions

1. Please describe the importance of embryo assessment.
2. Please explain why traditional, morphology-based embryo evaluation is not very efficient.
3. Please describe how amino acid turnover relates to developmental capacity of embryos.
4. Please define how amino acid profiling is different from other assays and how it may be used as a predictive marker for embryo selection.

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Microfluidics and Microanalytics to Facilitate Quantitative Assessment of Human Embryo Physiology

David K. Gardner, Philipp Reineck, Brant C. Gibson, and Jeremy G. Thompson

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Learning Objectives

- Limitations of conventional methods of embryo assessment
- Embryo viability is far more than how it looks
- Metabolism is linked to embryo viability
- Hyperspectral and FLIM microscopies can be used to quantitate autofluorescence, a measure of cellular metabolic activity
- Microfluidic devices, such as laboratory-on-a-chip, can be used to handle and quantitate submicrolitre volumes of culture media
- Laboratory-on-a-chip devices can be automated
- Future of IVF could incorporate both laboratory-on-a-chip and hyperspectral or FLIM microscopies, together with time-lapse image analysis

45.1 Introduction

Over the past decade, we have witnessed a worldwide movement towards single embryo transfer in human IVF. This has been driven by the adverse maternal and fetal consequences associated with multiple embryo transfer [1], and which has been facilitated by improvements in human embryo culture conditions, resulting in a significant increase in embryo implantation rates [2]. Together with improvements in embryo cryopreservation through vitrification, the cumulative success rates of cycles have improved overall. However, in order to reduce the time to pregnancy in each cycle, we need to be able to select the most viable euploid embryo for transfer. Identification of euploid embryos has been greatly facilitated by recent developments in comprehensive chromosome screening technologies, combined with trophectoderm biopsy from the blastocyst [3]. However, not all euploid embryos are viable, and hence there remains a need to quantitate embryo viability prior to transfer.

Since the birth of Louise Brown in 1978, we have relied almost exclusively upon morphology to assess the health of the preimplantation embryo. Over the past four decades, we have witnessed the development of elegant grading systems for all the preimplantation stages, and this approach has certainly aided in the deselection of embryos with abnormal phenotypic traits [4]. More recently, we have witnessed the introduction of time-lapse incubation systems, which have created the possibility to image embryos every few minutes, thereby increasing the amount of information we have regarding the times of key developmental events [5, 6]. This approach in turn is leading to the development of algorithms, further capable of improving embryo deselection [7, 8]. However, one can only glean so much information by looking at cells using basic light microscopy. Hence, it is important to consider novel technological means of assessing embryo physiology. We propose that by assessing physiological traits, such as nutrient utilisation and metabolic activity, or the production of embryo-specific factors, we will be able to identify the healthiest embryos within a cohort with increased accuracy for transfer.

45.2 Significance of Analysing Embryo Physiology

The preimplantation period is characterised by changes in embryo morphology and gene expression as the fertilised oocyte develops and differentiates into the blastocyst. Furthermore, during this period, the physiology of the embryo undergoes remarkable transformations, so much so that the pronucleate oocyte and the resultant blastocyst are as distinct in their metabolism as any two cell types in the body [9]. This in itself is a remarkable observation. Additionally, we are still unravelling the complexities associated with the metabolism of the preimplantation mammalian embryo [10–12]. What we have ascertained to date is that the pronucleate oocyte and cleavage-stage embryos are heavily dependent upon pyruvate, lactate and specific amino acids such as aspartate to support their unique metabolic functions, whereas the blastocysts have a high demand for glucose, as well as amino acids and vitamins [9, 13]. From a practical perspective, this means that the metabolic biomarkers of the preimplantation embryo will be different for each successive stage of development. Therefore, should a clinic decide to transfer embryos at the cleavage stages, then the measured metabolism will heavily reflect the quality of the oocyte. Should a clinic undertake blastocyst transfer, then the metabolic analysis will indicate that of the embryo proper. As the embryo develops and differentiates, the overall metabolic activity of the embryo increases. Therefore, there will be greater differences in the metabolic activity in the later stages of development, thereby making embryo selection using a quantitative measure of nutrient utilisation more realistic (Fig. 45.1).

To date, there have been few studies on the relationship between nutrient uptake and metabolism of the human embryo and the relationship with transfer outcome. Both animal and human studies show that a positive relationship between metabolic activity and embryo viability on days 4 and 5 of development exists [14–17]. Figure 45.2 shows clinical data obtained from the analysis of glucose uptake by individual human blastocysts prior to transfer. On both days 4 and 5 of development, there is very little overlap between the two populations of embryos (viable vs. nonviable), indicating that glucose uptake is a useful marker of viability in the post-compacted human embryo.

Such targeted and accurate measurements of specific nutrients and metabolites is made possible by enzyme-based assays and quantitative ultramicrofluorescence [18] and should not be confused with the attempts to assess human embryo viability utilising metabolomic platforms [19]. Technologies such as nuclear magnetic resonance (NMR)

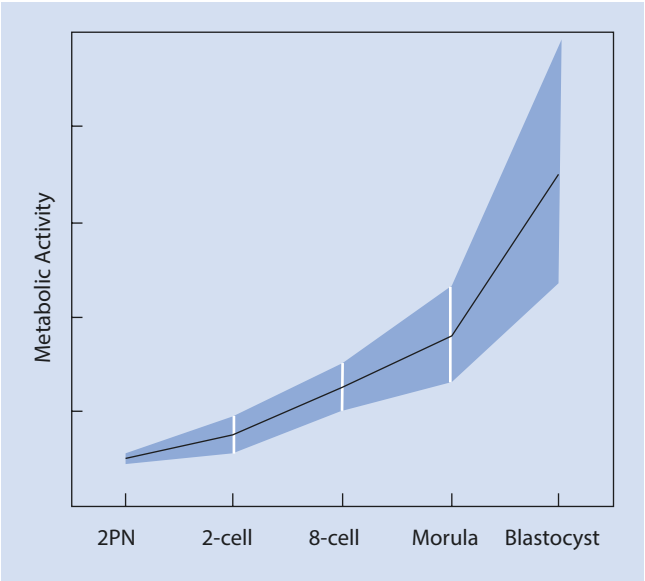


Fig. 45.1 Range of metabolic activities as the embryo develops through the preimplantation period. During the cleavage stages, the embryo has low metabolic activity; hence, there will be a small range of activities around the mean. In contrast, at the blastocyst stage, the embryo is metabolically at its most active, and further there exists more variation in embryonic cell number. Hence, at the blastocyst stage, there is a greater spread of metabolic activities, thereby making it more feasible to use metabolic activity as a selection criterion of embryos for transfer

imaging do not possess the sensitivity or resolution to work at the single embryo level. Raman and near-infrared (NIR) spectroscopies have been used to analyse human embryo culture media [20]; however, NIR spectroscopy is unable to quantitate the levels of specific nutrients but rather create spectra reflecting the relative abundance of metabolites in a sample. Although the latter approach was utilised to create a viability index of human embryos [21], this approach was not able to identify viable embryos in a prospective manner [22]. Nevertheless, research continues in these and other spectroscopy fields as such technologies rapidly advance [23].

45.3 Novel Microanalysis Techniques

All IVF labs are equipped with basic microscopes, such as dissecting microscopes used for visualisation and grading of oocytes and embryos, to advanced microscopes that provide enhanced contrast, such as phase contrast (especially useful for visualising spermatozoa), differential interference contrast, polarisation or Hoffman contrast optics. These latter optical systems are invaluable for visualising morphological features of cells, and are therefore highly effective tools to identify distinctive differences between gametes and embryos.

Fluorescence microscopy is found in many clinics because several fluorescent stains are particularly useful in the andrology laboratory for distinguishing live and dead sperm and morphological anomalies. Fluorescence microscopy is widely

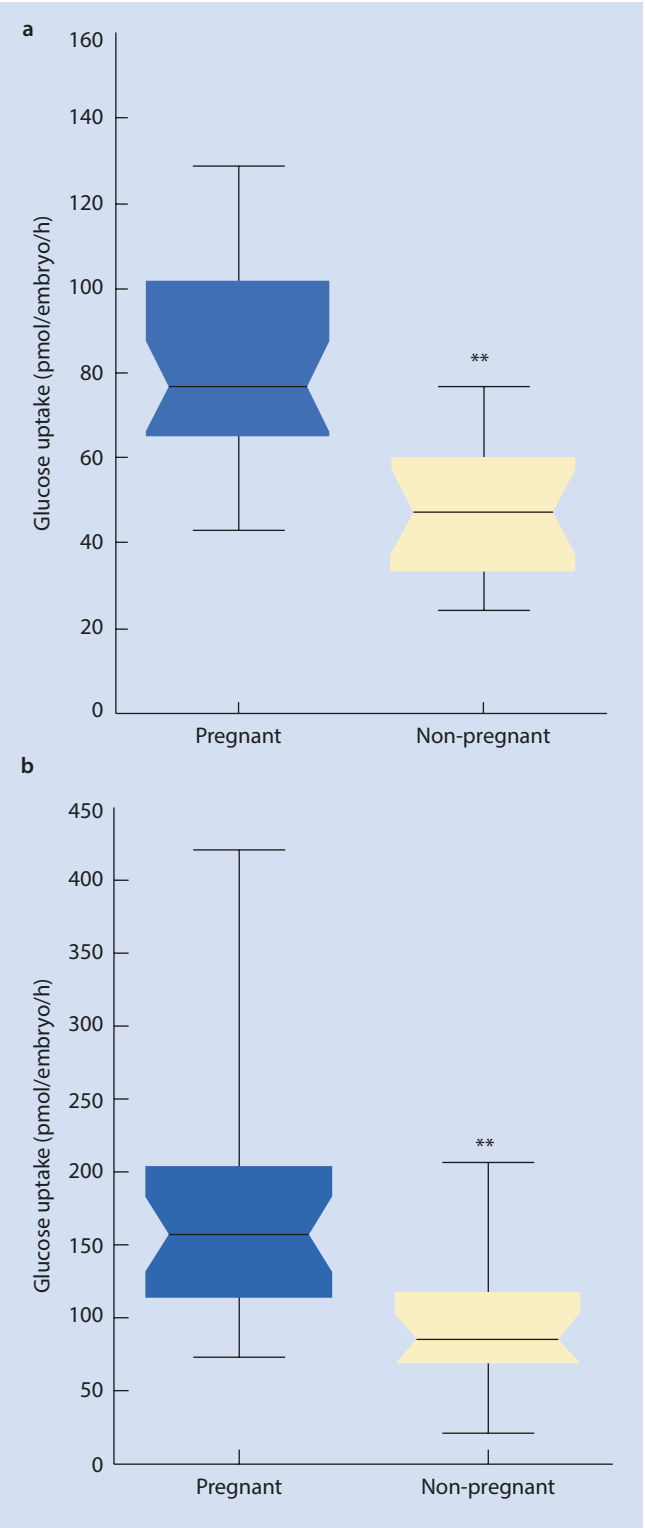


Fig. 45.2 Relationship between glucose consumption on days 4 and 5 of development and human embryo viability. **a** Glucose uptake on day 4 of embryonic development and pregnancy outcome (positive fetal heart beat). **b** Glucose uptake on day 5 of embryonic development and pregnancy outcome (positive fetal heart beat). Notches represent the confidence interval of the median, and the depth of the box represents the interquartile range (50% of the data), whiskers represent the 5 and 95% quartiles. The line across the box is the median glucose consumption. **, significantly different from pregnant ($P < 0.01$). Adapted from Ref [17]

used as a research and diagnostic tool. Its main advantage over many other microscopy techniques is that specific parts of a cell, such as a particular protein or organelle, can be identified and located in a fluorescence microscopy image with subcellular resolution. This is achieved by labelling a specific part of a cell with a fluorophore, which also enables very high image contrast. To image the sample, the fluorophore is 'excited' with the light of a specific wavelength (e.g. green), and only the light of longer wavelengths (e.g. red) emitted by the fluorophore is collected to create an image on an otherwise black background. Confocal fluorescence microscopy even allows the acquisition of these images in 3D with subcellular optical resolution. All fluorescence microscopy techniques are generally suited for live-cell as well as fixed-cell imaging. However, the relative high intensity of the excitation light, especially blue and UV light, can have adverse effects on live cells and embryos in particular [24]. The intensity and wavelength of light used for imaging embryos during their manipulation are important factors for maintaining the viability of preimplantation embryos in vitro.

Two-photon fluorescence microscopy allows 3D imaging of biological samples up to a depth of almost 1 μm at subcellular resolution and can be used to image live mammalian embryos [25]. It generally uses near-infrared light, which enables higher tissue penetration compared with visible light and minimises light-induced tissue damage.

Autofluorescence refers to the natural fluorescence emission of light from endogenous biological structures and molecules upon light excitation. Autofluorescence is usually highly undesirable, since it creates a fluorescent 'background' in most biological samples, which reduces the image contrast created by the exogenous fluorescent label in an image. However, it may also create new opportunities to identify and image biologically important molecules based on their autofluorescence, many of which are involved in metabolism (see Table 45.1). Several studies have shown that metabolic activity can be investigated in oocytes and embryos through autofluorescence imaging and spectroscopy [26, 27]. The best-characterised autofluorescent metabolic molecules are NAD(P)H, which both fluoresce in blue upon UV-excitation, and FAD, which fluoresces in green upon blue light excitation. Both NADH and FAD participate in electron transport for oxidative phosphorylation, and the fluorescence ratio between the two is a measure of the cellular reduction-oxidation ratio.

Further advances in both fluorophore-mediated and autofluorescence microscopy have focussed on being able to distinguish very subtle differences in the fluorescence properties of closely related molecules. For example, small differences in fluorescence of bound- and free-NADH can be measured using fluorescence lifetime imaging microscopy (FLIM). The ratio between bound- and free-NADH is indicative of glycolytic or oxidative phosphorylation activity. FLIM was used to demonstrate the differences between mural granulosa and cumulus cell metabolism compared with oocyte metabolism within ovarian follicles in situ [28]. Another emerging fluorescence-based technique is hyperspectral autofluorescence microscopy. Most fluorescence microscopes utilise not more than four different excitation light wavelengths (colours), generated by different lasers or LEDs. However, with the development of lasers that generate continuously tunable excitation light from the UV to the near-infrared spectral range, or the use

Table 45.1 Excitation and emission maxima of endogenous cellular autofluorescence fluorophores

Endogenous fluorophore	Excitation maxima (nm)	Emission maxima (nm)
Amino acids		
Tryptophan	280	350
Tyrosine	275	300
Phenylalanine	260	280
Structural proteins		
Collagen	325, 360	400, 405
Elastin	290, 325	340, 400
Enzymes and co-enzymes		
NADH	290, 351	440, 460
NADPH	336	464
FAD and flavins	450	535
Vitamins		
Vitamin A	327	510
Vitamin K	335	480
Vitamin D	390	480
Vitamin B6 compounds		
Pyridoxine	332, 340	400
Pyridoxamine	335	400
Pyridoxal	330	385
Pyridoxic acid	315	425
Pyridoxal 5′-phosphate	330	400
Vitamin B_{12}	275	305
Lipids		
Phospholipids	436	540, 560
Lipofuscin	340–395	540, 430–460
Ceroid	340–395	430–460, 540
Porphyrins	400–450	630, 690

Adapted from Ref. [55]

of multiple LEDs, a broad range of autofluorescence signals can be investigated. This approach has enabled new insights into the heterogeneity of cellular metabolism between individual cells (including oocytes and embryos) within a defined environment. Its application to oocyte and embryo metabolomics remains a research tool at this stage, requiring answers to questions such as light toxicity and the clear identification of autofluorescent molecules. However, there is now good evidence that, in conjunction with image-analysis, hyperspectral autofluorescence can distinguish between embryos cultured in different environments [29, 30].

45.4 Why Has Metabolic Analysis of Embryos Not Been Applied Clinically?

Two principal reasons why analysis of individual human embryos has not been widely applied clinically are (1) there are no completely noninvasive intracellular metabolic assays available, and (2) the technologies to quantitate single embryo physiology accurately, such as ultramicrofluorescence assays and mass spectrometry of the media surrounding the embryo, used to analyse carbohydrates and amino acids, respectively, are both time-constrained and require equipment and skills not normally found in the IVF laboratory. Indeed, ultramicrofluorescence for single-cell analysis is restricted to a handful of laboratories worldwide. Hence, its implementation has been restricted to research and pilot studies to date. It was envisaged several years ago that this problem would be overcome through the development of microfluidic devices, capable of manipulating and analysing sub-microlitre samples of human embryo culture medium (Fig. 45.3) [31]. In the intervening decades, such laboratory-on-a-chip technologies have developed rapidly, and two proof-of-principle devices have been developed, each capable of performing metabolic analysis of single embryos [32, 33]. So, what are laboratory-on-a-chip technologies? In particular, what is the relevance of microfluidics to human embryo diagnostics?

45.5 Introduction to Microfluidic Systems: Laboratory-on-a-Chip

Microfluidic devices are capable of handling fluids in sub-microlitre volumes and take advantage of the physical differences of fluids between macro- and microscales. In the sub-microlitre scale, fluid flow is linear, i.e. nonturbulent, and through the use of appropriate on-chip valving, fluids can be moved and measured with remarkable accuracy [32]. Given the small volume of the human embryo, ~5 nl, this ability to quantitate small volumes of fluid makes microfluidic devices ideally suited for single embryo analysis. Further, the ability to deliver volumes accurately in this range ensures the accuracy of analysis. An exciting prospect for microfluidic devices for embryo analysis is that analytical technologies are already available that can be incorporated into chips. Micro-devices have been developed to analyse nucleic acids, proteins and peptides, amino acids and small metabolites [34–36]. A variety of technologies are applicable for integrated noninvasive analysis of the metabolism of embryos [37]. Furthermore, on-chip imaging can already produce images with resolution below 1 μm [38].

An attractive attribute of microfluidic devices is that their operation can be automated. Automation has the advantages of removing human error and facilitates assessment and manipulation of gametes and embryos based on optimal developmental stage of the embryo, rather the work schedule of a busy IVF laboratory. It is known that embryos

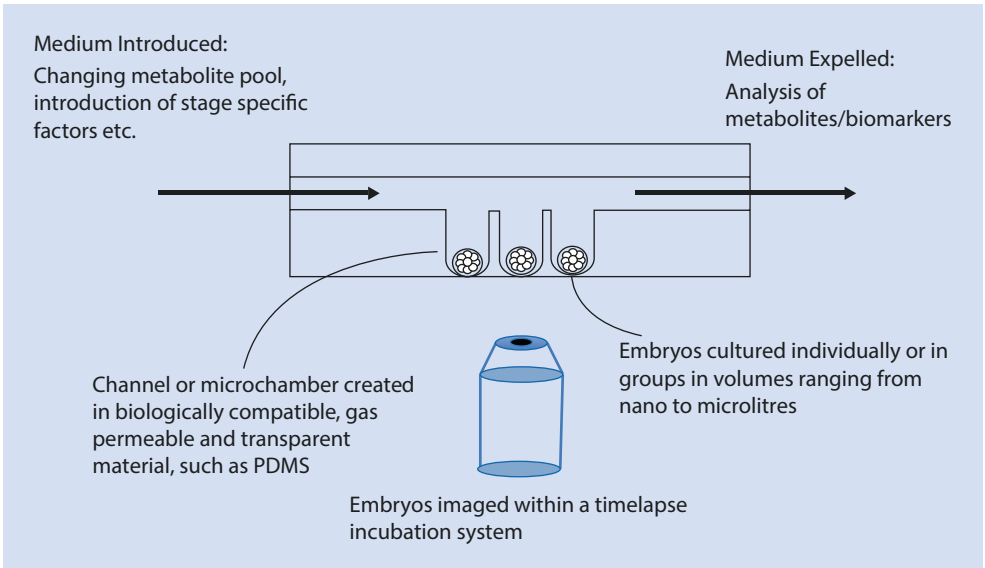


Fig. 45.3 Schematic of an embryo culture system for perfusion culture and analysis of biomarkers. Culture media are continuously passed over the embryo(s). The composition of the culture media can be changed according to the specific requirements of each stage of the embryonic development. Toxins, such as ammonium, are not able to build up and impair embryo development, while more labile components of the culture system are not denatured. Samples of the culture media can be removed for biomarker analysis. (Adapted from Ref. [31])

within a cohort show differences in developmental rates, even when they are fertilised at the same time. It is feasible that some of the biological variability in analyses can be removed if embryos are analysed according to precise developmental staging rather than based on the time post-fertilisation.

Microfluidic fabrication uses highly specialised and complex engineering methodology, and devices can be made using a number of different materials. Methods available include photolithography, soft lithography, laser machining, micromilling, bead blasting, chemical etching, wax printing and hot embossing. All of these are essentially surface modification techniques capable of creating high-resolution features, with micro- and nano-scale width and depth precision and with high reproducibility. Devices for biological uses have been fabricated from materials including borosilicate glass, light-sensitive films (photoresists), polymers (e.g. poly(dimethyl)siloxane (PDMS), polycarbonate (PC), poly(methyl-methacrylate) (PMMA)), teflons, waxes, shrink-wrap films and even paper-based materials. Most microfluidic devices for biotechnology applications have been made from glass [39] or from a polymer, typically PDMS [40].

Poly(dimethyl)siloxane is a silicon-based elastomer and optically clear, and devices are typically designed using photolithography and soft lithography, in which liquid polymer is typically poured over a rigid mould (master) produced as the inverse of the channel layout. The polymer is then cured by baking, and mounted on glass or other planar surfaces. This process is considerably cheaper than that for glass. Once the master has been prepared, the same master can be reused to make multiple single-use or minimally reused device copies. Drawbacks of PDMS for biological device applications include its reactivity to organic solvents, heat sensitivity and porosity. This latter property can promote reactions with aqueous samples contained within the device, such as small molecule absorption into the bulk material [41] or release of trace amounts of PDMS monomers and oligomers [42] which may confound mass spectrometry studies. However, in its favour, the elastomeric properties of PDMS make it highly suited for pneumatic valving, and it has been this which has been used to create analysis chips for individual preimplantation embryos [32].

45.6 Lab-on-a-Chip Analysis of Individual Preimplantation Embryo

In order to quantitate the utilisation of nutrients by single mouse embryos, a microfluidic device was created capable of measuring metabolites in spent culture medium (Fig. 45.4) [32]. This automated device can determine the concentration of glucose, lactate or pyruvate in just a few nanolitres of culture medium in 20 s. Particularly attractive devices for in-line monitoring of metabolites are biosensors that convert binding or a chemical reaction of a substrate on the surface of a sensor to an electrical signal [43]. These sensors can be fabricated very inexpensively using thick-film printing and can be developed to detect a wide variety of analytes. With the tremendous amount of ongoing research in microanalytics, we expect that even better technologies will emerge in the near future (see the following section).

45.7 Integration of Future Technologies into the Clinic

In research laboratories around the globe, new biomedical imaging and sensing technologies are advancing rapidly, driven by the clinical and scientific need for ever more sensitive measurements in living organisms at higher resolution. Image analysis approaches, such as grey-level co-occurrence matrix analysis, have been combined with artificial intelligence systems for embryo diagnostics [44, 45]. Conventional time-lapse images can be analysed using neural network algorithms to aid and improve the embryo selection process [46]. Fluorescence imaging and sensing, especially with near-infrared light, holds great potential for the development of novel and completely noninvasive embryo screening platforms. A prerequisite for this and all other light-based technologies is a better fundamental understanding of the effect of light itself on the physiology and viability of embryos.

Another frontier is the use of novel nanoscale materials for imaging and sensing in biology and medicine in general and embryology in particular. These so-called nanomaterials include FDA-approved molecules like fluorescein that are used today in the clinic as well as many different types of nanoparticles that are about 10,000 times smaller than an oocyte. Some of these new materials may enable next-generation imaging and sensing technologies through their unique physical and chemical properties. Nanomaterials that show stable fluorescence in the near-infrared spectral region [47] or that have the ability to detect vital ions or metabolites with high precision and sensitivity [48] are two examples. However, the effect of nanomaterials on biological systems in general and embryos in particular remains a major hurdle. Even biocompatible materials can affect cell stress, signalling processes or gene expression [49], which necessitates a better understanding of the underlying mechanisms. To date, several nanoparticles have been approved by the FDA or the EMA for clinical use [50]. On the other hand, no nanoparticles are approved for use in stem-cell therapies by the FDA. Preliminary evidence suggests that nanoparticles may be a useful tool for an improved assessment of embryos, such as the self-illuminating quantum dots for noninvasive bioluminescence imaging of mammalian gametes [51]. But this approach has not yet been established as safe. However, it has been demonstrated that the injection of ligand-free gold and silver nanoparticles into murine embryos has no impact on preimplantation

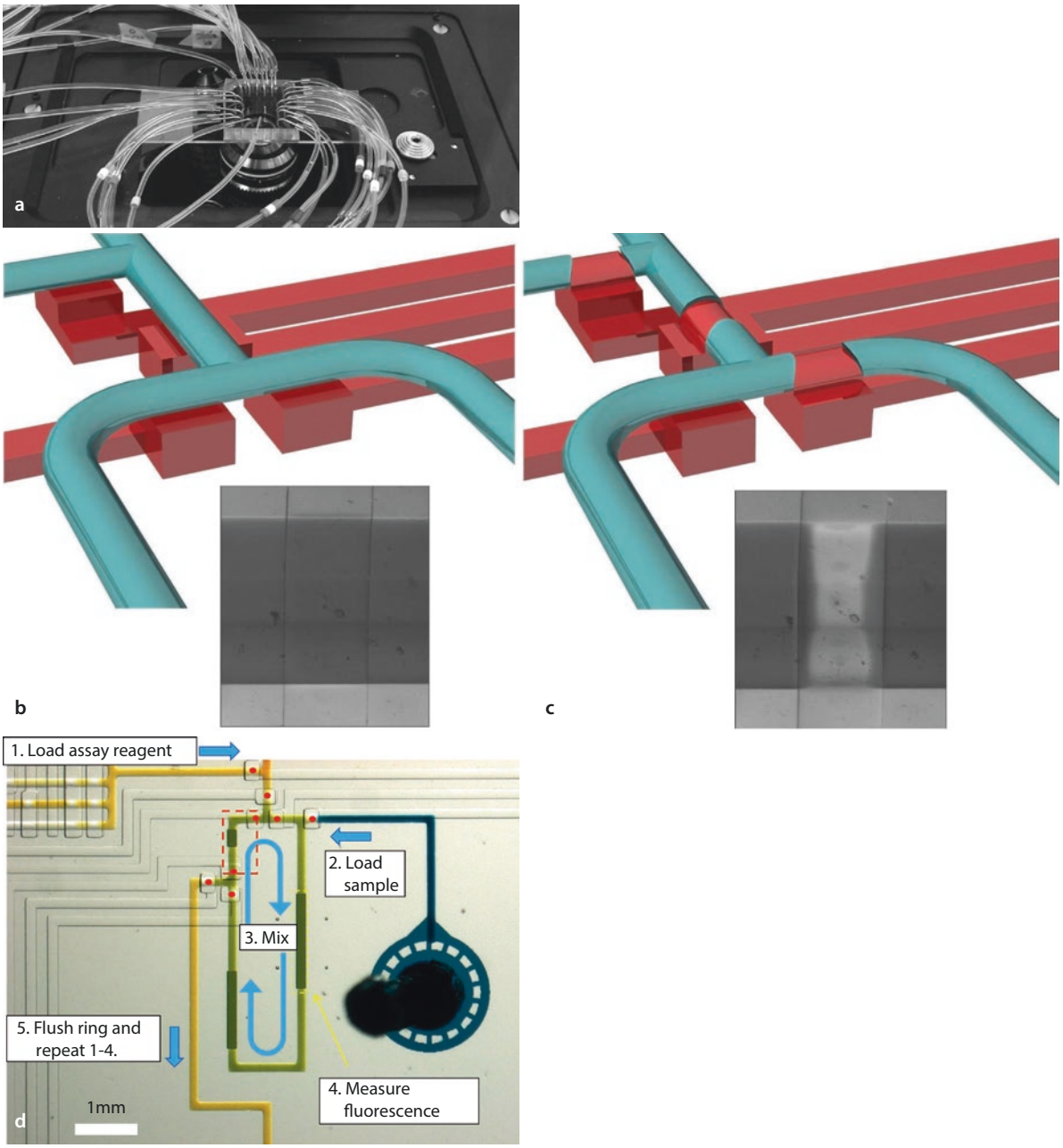
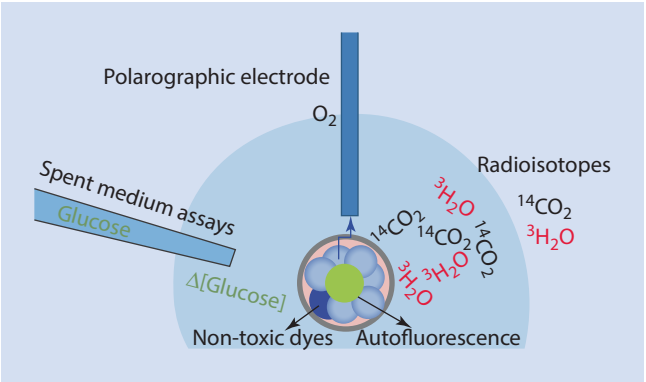


Fig. 45.4 Microfluidic device for determining the concentrations of metabolites in the culture media. **a** Image of a microfluidic device/chip, prepared for the analysis of embryo culture media [32]. The microfluidic circuits are prepared out of poly(dimethyl)siloxane, an elastomer, that facilitates the creation of pneumatic valving in order to move accurately volumes in the nanolitre range. **b**, **c** Computer-generated rendering of the microfluidic device, illustrating in 3D how the microchannel flow is controlled. The overall device format **b** shows individual fluid flow channels (blue) lying above a layer of control channels (red), which are driven pneumatically. Flow channels are a combination of rectilinear and rounded cross sections, with channel width uniformly around 100 microns. Red boxes represent valves that make contact with the blue flow channels from beneath, which appear in high density in the grid regions, representing the main control interface. A blow-up of a channel T-intersection **b**, **c** shows how each channel branch is positioned over three individual valves, the blind ending of a control line. When valves are open **b**, no hydraulic pressure is in the line, and fluid can move freely in the flow channel (inset micrograph of a valve from a PDMS device, viewed from above). To close a valve, the hydraulic pressure in the control line is increased, pushing the channel floor upwards and preventing fluid flow in that region (inset photo with lighter region is where flow is blocked) **c**. **d** A multiplexor with 10 input channels for automated loading of assay cocktail and standards. To the right is a sample-loading port where approximately 100 nl of culture medium is loaded manually. By automatically activating pneumatic valves (marked with red dots) in defined sequences, 9/10 of the mixing circuit with assay cocktail and 1/10 of the ring with sample (section of the mixing ring outlined with the red dashed line) is filled. Sequential firing of the valving within the mixing circuit then mixes the samples. The NAD(P)H fluorescence of the fluid within the measurement chamber (highlighted by dashed yellow lines) is then determined using an automated fluorescence scope equipped with a photomultiplier tube. The sample port and mixing circuit are then flushed clean, another culture medium sample loaded and the sequence repeated. To see this procedure in real time, go to the supplementary video file included with reference number [32]

development [52]. The field of nanotoxicology is a rapidly growing field of fundamental and applied research. Its findings will be decisive for the future development of nanomaterial-based technologies that are safe for clinical use. Many of the nanomaterials that are used in the clinic today are administered systemically. An alternative and in some cases more realistic approach is the integration of nanomaterials into new microscopic imaging and sensing devices such as optical fibres. These may allow for high-precision chemical sensing, optical imaging and real-time monitoring of the embryo metabolism during development without the need for a global exposure of the biological system to the nanomaterial (■ Fig. 45.5 and ■ Table 45.2).

In conclusion, real-time monitoring of embryonic physiology is soon to come and will revolutionise how embryos are cultured. Culture conditions will no longer be static or invariably dynamic but instead will be constantly adjusted in response to the embryo's changing needs [31, 53]. Real-time read out of embryonic physiology will be tremendously valuable both for optimising and monitoring culture conditions and for assessing the health and developmental potential of the embryo [56].



■ **Fig. 45.5** Different methodologies that are currently used to measure the metabolism of early embryos. Radioactive isotopic energy substrates, such as [U-14C]- or [5-3H]-glucose, were invaluable research tools, but not suitable as noninvasive markers for subsequent embryo transfer. The same applies to dyes and stains, even if they are relatively nontoxic. Sampling the media surrounding embryos for subsequent analysis, usually in nanolitre volumes, or direct measurement of O_2 consumption using electrodes, is currently the safest and most clinically applied. Autofluorescence is a direct yet virtually noninvasive measure of cellular metabolism. Clinical application requires further assessment about its safety and reliability. (From Ref. [54] with permission)

Review Questions

1. What are the limitations of morphological assessment?
2. Which nutrients are related to embryo viability?
3. What is laboratory-on-a-chip?
4. What types of novel microscopies offer potential means of assessing embryo physiology?
5. Are the technologies now ready for automated embryo culture and analysis?

■ **Table 45.2** Near-infrared fluorescent nanomaterials. The table summarises some of the basic optical properties of important nanoparticle types

	Excitation range (nm)	Emission range (nm)	Fluorescence lifetime	Photostability
Quantum dots	700–1300	700–1500	20 ns–5 μs	High
Polymer dots	500–800	650–1200	≤1 ns	Medium
Nanodiamonds (NV)	520–580	650–750	10–30 ns	Very high
Organic dyes	600–800	650–1100	<1–6 ns	Low
Carbon dots	500–650	650–650	≤10 ns	Medium
Gold clusters	500–650	650–850	3–800 ns	Medium
Carbon nanotubes	700–1300	1000–1500	≤1 ns	High
Graphene oxide	400–650	650–700	≤1 ns	Medium
UCNPs	980	650–800	>100 μs	High

Information adapted from Ref. [47]

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Assisted Hatching of Human Embryos for Successful Implantation

Matteo A. Avella, Kristine A. Milne, Shagufta Dawood, Adam Dawood, and Michael J. Tucker

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Learning Objectives

- Structure and function of the *zona pellucida* during fertilization, embryo development, and hatching
- Assisted hatching: concept and techniques
- Clinical cases that benefit from assisted hatching
- Optimization of noninvasive and effective assisted hatching procedures

46.1 The Zona Pellucida Mediates Gamete Recognition and Protects the Embryo Until Implantation

Successful monospermic fertilization defines the onset of proper embryo development and requires gamete recognition and a block to polyspermy. Both necessary biological processes are mediated by the *zona pellucida* (ZP), an extracellular glycoprotein matrix that surrounds mammalian ovulated eggs. Mouse and human ZP appear different in thickness (mouse ZP ~7 μm thick; human ~16 μm), surround oocytes of significantly different sizes (mouse oocyte ~80 μm diameter; human 120 μm), and pertain a comparable, but still different, molecular composition. Mouse and human proteins are encoded by four genetic loci. The mouse ZP is composed of three glycoproteins, defined as ZP1 (526 aa, 120 kD), ZP2 (599 aa, 120 kD), and ZP3 (329 aa, 83 kD) [1, 2], while mouse *Zp4* is a pseudogene that does not express the cognate protein [3]. Human ZP is composed of four glycoproteins, defined as ZP1 (528 aa, 100 kD), ZP2 (602 aa, 75 kD), ZP3 (328 aa, 55 kD), and ZP4 (444 aa, 65 kD) [3, 4].

The structure of the ZP has been widely studied in the mouse [5, 6] and other experimental models [6]. In mouse, the three ZP proteins form a three-dimensional *zona* matrix by non-covalent interactions. At the C-terminal region, each protein has a "ZP domain" of ~260 aa [6, 7] and a cytoplasmic tail [8] that ensure protein incorporation into the extracellular matrix of the *zona*. The ZP domain contains 8–10 conserved cysteine residues and is divided into *N*-terminal "ZP-N" and C-terminal "ZP-C" subdomains [5]. The structure of each protein is stabilized by intramolecular disulfide linkages, whereas ZP1 only provides intermolecular disulfide bonds that form homodimers within the *zona* [2, 9] and further stabilize the three-dimensional structure of the matrix [10].

Although the molecular architecture of each ZP protein has been extensively described, there has been much controversy over the characterization of the oocyte zona ligand and the identification of the cognate sperm receptor, necessary for sperm binding to the ZP. Early studies in mice had suggested that glycans on ZP3 mediate sperm-zona interaction. In particular, back in the 1980s, using soluble, SDS-PAGE-purified, and renatured *zona* proteins in an in vitro competitive sperm-binding assay with ovulated eggs, ZP3 was reported to inhibit sperm-ZP binding in vitro with the highest efficiency. Indeed, no inhibitory effect of sperm binding to ovulated eggs was observed with renatured ZP1 or ZP2; therefore, ZP3 was reported as the *zona* ligand for mouse sperm binding [11]. Further studies made this model of gamete recognition more precise, invoking *O*-glycans [12] attached to Ser^{332} and Ser^{334} of ZP3 as the zona ligands for sperm binding [13]. This model envisioned that after fertilization, these glycan ligands would have been released by a putative glycosidase exocytosed from egg cortical granules to prevent sperm binding to fertilized eggs [12]. However, phenotypes observed in genetically engineered mice were not consistent with this model. In particular, no carbohydrates were found attached to Ser^{332} and Ser^{334} in native mouse ZP [14], and even after mutating these sites to prevent glycosylation, fertilization was not compromised [15].

To define which ZP protein mediates sperm-egg binding, an alternative approach was the systematic use of mouse genetics to test the role of each individual ZP protein in gamete recognition. Using knock-out (KO) gene technology, each ZP protein has been successfully ablated in transgenic mice; females lacking ZP1 present a more loosely organized *zona* matrix and show decreased fecundity, although they are still fertile, which shows ZP1 not to be necessary for gamete recognition [10]. On the other hand, female mice lacking either ZP2 [16] or ZP3 [17, 18] present a more severe phenotype, with no ZP surrounding ovulated eggs. These female mice are infertile as their *zona*-free eggs are resorbed into the epithelial lining of the oviduct [19, 20]. Thus, ZP1 is not necessary for gamete recognition and fertility, but the absence of a ZP matrix in the $Zp2^{Null}$ and $Zp3^{Null}$ mice precludes the possibility to test the role of either ZP2 or ZP3 in gamete recognition. As these loss-of-function assays were not informative on gamete recognition, this was circumvented by taking advantage of the stringent specificity of sperm binding across taxa.

In fact, although the primary structure of secreted mouse and human ZP1 (71%), ZP2 (62%), and ZP3 (71%) is quite conserved, human sperm bind only to human and neither bind to the mouse ZP [21] nor fuse with mouse eggs [22]. Therefore, with this taxon specificity in mind, we have established transgenic mouse lines in which human ZP1, ZP2, and ZP3 replaced the endogenous mouse proteins, and we inseminated eggs from each individual line with fertile human sperm. Human sperm bound to the chimeric mouse-human ZP only in the presence of human ZP2 [23, 24], but not in the presence of human ZP1, ZP3 [23], or ZP4 [25]. To complement this gain-of-function assay, we have established loss-of-function assays to testimony that ZP2 was not only sufficient but also necessary to support sperm binding to the ZP. In mice with a ZP formed by four human proteins and lacking the three endogenous mouse proteins, human sperm bound and penetrated the zona matrix. However, in the absence of human ZP2, these mutant zona could not support human sperm binding, and female mice were infertile [24]. Moreover, mouse *zonae* in which mouse ZP2 was replaced with human ZP4 could not support mouse sperm binding, and these females were also infertile [24]. The gain- and loss-of-function assays document that ZP2 rather than ZP3 is the zona ligand for mouse and human sperm binding to the ZP, which is necessary for fertilization. Moreover, by establishing

transgenic mouse lines that express a truncated mouse ZP2 that lack the *N*-terminus of ZP2 in place of the native isoform, we found that mouse sperm must bind to the *N*-terminus of ZP2 to successfully fertilize the egg. Indeed, eggs lacking the ZP2 *N*-terminus cannot support sperm binding, and female mice are infertile [24]. Finally, by establishing chimeric human/mouse and mouse/human ZP2 in transgenic mice, we refined the gamete recognition domain for human sperm at the *N*-terminus of human ZP2.

Following fertilization, the block to polyspermy ensures monospermic fertilization, which is imperative for proper embryo development. This block includes putative or characterized molecular modifications to the ovulated egg that prevent supernumerary sperm: (1) to fuse with the egg plasma membrane; (2) to penetrate the ZP; and (3) to bind to the ZP [26, 27]. Block to fusion was found not to be associated with electrical potential changes [28, 29], and the molecular mechanisms regulating this block still remain to be defined [30]. While the block of ZP penetration has not been molecularly characterized yet, the block to ZP binding has been recently well described. Sperm can bind to the ZP surrounding unfertilized eggs, but cannot bind to the ZP of fertilized eggs or the 2-cell embryos, and this has been originally associated with an imprecisely defined "*zona* hardening" mechanism. The only documented biochemical modification of the ZP upon fertilization is the proteolytic cleavage of ZP2, after which the two fragments remain disulfide bonded [31]. This cleavage is due to the activity of a cortical granule protease defined as ovastacin [32] specifically localized in the cortical granules. After fertilization, the cortical granule exocytosis releases ovastacin, which cleaves ZP2 at the *N*-terminal region [33]. As a consequence, sperm are no longer able to bind to the ZP [33]. However, when the postfertilization cleavage of ZP2 is genetically prevented, either by mutating the ZP2 cleavage site [15] or ablating the gene encoding OVASTACIN [33, 34], sperm can bind de novo to the ZP surrounding fertilized eggs and 2-cell embryos. Of note, OVASTACIN is encoded by the *Astl* gene, which belongs to the Astacin family. In fish, *Astl* genes encode for the hatching enzymes [35] that assist the fish larvae to escape from the egg envelope upon completion of embryonic development. In mammals, this gene family seems to have acquired a different function during early embryo development, as mediator of "*zona* hardening" right upon fertilization [33], whereas other still-to-be defined mechanisms seem to assist natural embryonic hatching, which is required for appropriate embryo implantation.

For successful implantation and a healthy and safe pregnancy, the timing of hatching through the ZP is of crucial importance. While progressing toward the uterus, the forming blastocyst expands, stretching the ZP, which finally appears thinner (~3 μm) than at the time of fertilization (16 μm) [36]. This has been proposed to be due to the activity of a Na^+/K^+-ATPase plasma membrane pump of the trophoblast cells that pumps sodium into the blastocoel, which in turn recall water by osmotic pressure, leading to the enlargement of the blastocoel [37] which thereby stretches the ZP [38]. At this developmental stage, the ZP is necessary to impede the blastocyst adhering to the oviduct walls, thus blocking an ectopic (or tubal) pregnancy. However, once in the uterus, the embryo must escape from the *zona* matrix in order to implant into the uterine lining.

Studies to describe mammalian implantation have been performed in the mouse. In vitro the mouse blastocyst hatches from the *zona* by breaching through a ~3 μm hole, anywhere in the ZP [39]. It has been proposed that a proteinase expressed in the trophoblast cells, defined as strypsin/ISP1 [40, 41], controls the blastocyst's hatching [41] by digesting the entire ZP. Indeed, the inhibition of strypsin expression using antisense oligodeoxynucleotides against *Isp1* mRNA resulted in improper hatching and implantation was affected [41]. It still remains to be defined what protein(s) in the ZP are targeted by the lysis activity of ISP1. Moreover, it would be informative to confirm this mechanism in genetically modified mice in which conditional ablation of *Isp1* in the embryo would result in impairment of in vitro hatching and subsequent implantation failure upon Δ*Isp1* blastocyst transfers in the uterus of recipient female mice.

46.2 Assisted Hatching: Historical Background and Current Clinical Applications

46.2.1 History of Assisted Hatching

In the field of assisted reproductive technology (ART), the procedure of assisted hatching (AH) consists of the chemical/mechanical manipulation of the ZP before embryo transfer to facilitate the escape of the embryo from the ZP and its subsequent implantation in the uterine lining [42, 43]. Within the realm of AH, several techniques were developed over the past few decades [44], which include chemical drilling of the ZP with acidic Tyrode's medium; ZP thinning using acidic Tyrode's; mechanical piercing of the ZP using a microneedle, known also as partial zona dissection (PZD); mechanical expansion of the ZP via injected hydrostatic pressure; carving a hole in ZP via piezoelectric pulses; and laser-assisted *zona* drilling.

Early reports on the application of artificial manipulation of the ZP using acidic Tyrode's were performed in mice in 1986 [45]. Although the primary goal of these proof-of-concept studies was to facilitate mouse fertilization in vitro, the authors obtained positive results on implantation as well [45]. In 1988, a successful pregnancy was achieved in the human when PZD was also performed to facilitate sperm penetration [46]. From these pioneer studies, it was hypothesized that an artificial manipulation of the ZP could have assisted the embryo to escape from the ZP, which in turn would have resulted in higher implantation rates. Indeed, in 1990, a subsequent compelling study reported a significantly higher implantation rate (n = 8/15; 53%) when the *zona* of 8-cell embryos was mechanically pierced with respect to the non-manipulated control (n = 3/15; 20%) [47]. In subsequent studies [48], artificial ZP manipulation was applied to 4–8

cell embryos following in vitro fertilization (IVF) from patients with basal FSH levels (≤15 mIU/ml) that presented a ZP thickness ≥15 μm and exhibited low implantation prospects. Cohen and colleagues concluded that the reason behind the lower implantation and pregnancy rates observed could have been the restriction of a thicker ZP that may have impeded normal embryo escape. To address this hypothesis, embryologists applied topically, over a ~30 μm area of the ZP, acidic Tyrode's solution to chemically drill a hole in the ZP and reported upon transfer a significant increase of implantation rate (n = 70/278; 25%) compared to the non-manipulated controls (n = 51/285; 18%). Of interest, embryos presenting a thinner zona (average thickness ≤13 μm) did not benefit from the procedure; instead chemical AH had adversely impacted embryos resulting in 25% (n = 5/25) implantation compared to the non-manipulated embryos (n = 13/26; 50%) [48].

With time, alternative mechanical approaches were developed and adopted. Initial mechanical techniques for AH involving PZD using a microneedle to perforate the ZP resulted in inconsistent openings [47, 49]. AH was also accomplished using a piezo-micromanipulator where piezoelectric pulses producing vibratory movements are repeatedly applied to drill a hole in the ZP [50]. This approach has proven effective on "good-quality" embryos (embryo grade based on Veeck's classification [51]), resulting in a better implantation rate (n = 13/67; 19.4%) compared to the non-hatched controls (n = 4/68; 5.9%), but had no significant effect on low-quality embryos [50]. Another interesting and more recent approach involved simulating the natural expanding effects of blastocysts on the ZP by injecting HTF-HEPES medium into the perivitelline space of 8-cell stage embryos to induce internal hydrostatic pressure, which in turn would have assisted a more physiological escape of the embryo from the zona [52]. This novel approach resulted in a relatively total low implantation rate (n = 25/178; 14%), although significantly higher when compared to the non-hatched control (n = 14/90; 7.4%).

Of all the above-listed techniques, laser AH has been reported to be the most effective and noninvasive procedure [44, 53]. Laser beams offer high precision and control when thinning or drilling the ZP [44]. As of today, laser AH remains the most popular technique as it minimizes overall risk of visible injury to the embryos [44], and although it is not recommended as a routine clinical procedure, it has been reported to improve pregnancy and implantation rates in poor prognosis patients [54].

46.2.2 Clinical Applications

Clinical cases that might benefit from AH techniques include embryos that have less chance to normally implant due to a suggested failure to properly expand/contract and subsequently fail to reduce the thickness of the ZP down to ~3 μm at the blastocyst stage [55]. Advanced maternal age, in vitro culture conditions, and cryopreservation procedures have been associated with an increased number of embryos presenting low elasticity of the ZP, a still molecularly uncharacterized *zona*-hardening effect, and an impairment of normal ZP thinning [56].

46.2.2.1 Advanced Maternal Age and Increased Zona Thickness

Embryos from infertile female patients of 38 years of age and above present sharply declining developmental performance and significantly reduced fertility potential. Many factors have been suggested to be the cause of this decrease in fertility including diminished ovarian reserve and oocyte quality and in addition embryos presenting with aneuploidy and/or poor morphology. One more factor proposed is a still biochemically uncharacterized *zona* hardening affecting poor morphology embryos [57] that might restrict and prevent the blastocyst from escaping the ZP. While there is no molecular evidence showing that these embryos present a prematurely cleaved ZP2 or any other molecular signs of *zona* hardening, recent studies report that the application of laser AH improves embryo escape and implantation rates (18.36% vs. 11.36% of the non-hatched embryos) [56] in this cohort of patients. Other than advanced maternal age, patients with an elevated day 3 follicular stimulating hormone (FSH) level ranging from ≥7 mIU/ml to ≥15 mIU/ml are likely to show poor ovarian response [58]. Under this scenario, early studies have showed a higher implantation rate upon AH on 4–8-cell embryos from patients with elevated FSH basal levels (AH, 26% of 38 embryos; control, 10% of 40 embryos) [48].

Excessive ZP thickness has been a matter of concern and has been observed also in female patients less than 38 years of age. However, two studies have shown no correlation between the ZP thickness on embryo development and implantation [59, 60], and indeed when AH (performed by acidic Tyrode's solution) was applied to embryos with a ZP thickness ≥13 μm, implantation rates were not improved [61].

46.2.2.2 Cryopreserved Embryos

Upon cryopreservation, it has been proposed that a biochemically uncharacterized ZP hardening occurs [62, 63]. In early studies, we and others have documented a slight improvement of implantation rates upon AH performed on cryopreserved embryos [64, 65]. The first report included 125 embryos which underwent cryopreservation; AH was performed on 64 thawed embryos, 10 of which (16%) resulted in viable implantation, whereas only 6 out of 64 total in the unhatched control group (9%) successfully implanted [64]. Comparable results were observed in a subsequent study where AH was performed on 269 cryopreserved and thawed embryos, 37 of which implanted upon transfer (13.7%), whereas only 15 out of 284 (5.3%) implanted successfully without AH [65]. More recently, similar implantation rates were observed using AH performed by acidic Tyrode's solution applied to 136 cryopreserved embryos, which resulted in a higher implantation rate (11.4%) than the non-hatched control (5.8% of 117 embryos) [66]. Even *zona* thinning was having a positive

impact on the implantation rate of cryopreserved embryos, resulting in a 16.7% implantation rate versus 7.3% of the "non-thinned" control (200 total frozen embryo cycles) [67].

When in frozen embryo transfer cycles, AH seems to benefit the implantation rate of the cryopreserved embryos, studies which analyzed hundreds of clinical cases of fresh cycles have concluded that there are no strong evidence supporting a necessary role for AH in improving fertility outcomes (extensively reviewed in [68, 69]). However, with novel and emerging time-lapse video imaging, a high heterogeneity in the human embryos populations appears. We and others have observed different developmental patterns and implantation potential, even among embryos from the same cycle [70–72], which could have been the result of heterogeneity in embryonic genetics and physiology. Although further studies are needed to explore this heterogeneity, it might be reasonable to assume that although presenting similar characteristics (comparable age, morphology, culture conditions, etc.), different embryos might perform differently in developmental outcomes upon AH.

46.2.2.3 Repeated IVF Failure

Recurrent failed IVF cycles may be due to several causes which include reduced endometrial receptivity and endometriosis, embryonic developmental defects, genetic mutations inherited from the parental gametes, and aneuploidy. Oftentimes, the cause of IVF failure is unknown, and recurrent idiopathic IVF cycle failure is correlated with IVF failure in subsequent cycles.

46.3 Risks Associated with Assisted Hatching: Comparative Studies on Humans and Animal Models

Studies on animal models may inform clinicians on how the artificial induction of embryo hatching from the ZP may impact the cellular physiology of the embryo and the subsequent fertility outcome. Animal models offer an almost unlimited number of embryos and reagents that can be used as a platform to investigate the best conditions for assisted hatching and to provide insights on the possible physiological side effects that have been associated with this procedure.

A number of studies [73–75] in mouse have indicated conditions to perform a most effective assisted hatching of the mouse early embryo. While the use of acidic Tyrode's [76] solution has been largely replaced by less-invasive laser-assisted technologies [46], the impact of laser photoablation on the blastomeres adjacent to the ZP has been the matter of concern [77]. To avoid involuntary laser ablation of valuable blastomeres, some studies evaluate the possibility of thinning versus complete opening of the ZP and the pattern of embryo hatching on the procedure's outcomes, which include the development of the inner cell mass (ICM) and the rates of hatching, implantation, and pregnancy. In carefully designed studies, investigators have compared laser-assisted *zona* thinning with complete laser-assisted *zona* opening, using naturally conceived, genetically inbred, International Cancer Research (ICR) mouse embryos. As a negative control, intact-*zona* embryos were adopted [78, 79]. Assisted hatching was performed at the 8-cell stage followed by embryonic development and hatching pattern recording by time-lapse video imaging. Of interest, "*zona* thinning" only slightly improved the hatching rate of blastocyst, resulting in 39.3% of embryos hatched, which was comparable to the hatching rate of the negative control (33.9%), consistent with clinical data obtained in humans (200 patients), showing that thinning of the ZP (when performed chemically) does not improve implantation rate [80]. Indeed, a complete opening of the mouse ZP resulted in a 94.4% hatching rate. Another study used embryos from the same mouse strain (ICR), but assisted hatching was performed at the 2-cell stage. The results showed laser-assisted *zona* thinning to be more efficient than the previously described study, obtaining a similar number of hatched-blastocysts compared to laser-assisted *zona* hatching. One possible interpretation of the inconsistency of results between these two studies is that embryos may develop "better" in vitro if, since fertilization, they are not under the restriction of a constraining ZP.

Another compelling biological question that has intrigued the field in the past decades is the reason behind the ~2–12-fold [81, 82] increase in the incidence of monozygotic twinning in patients undergoing assisted reproductive technologies, particularly with assisted hatching, compared with natural conception. The phenomenon of monozygotic twins seems to be induced by physical, molecular, and mechanical factors, and it does occur in nature in several conventional and nonconventional animal models. For example, the 9-banded armadillo produces naturally identical quadruplets. These twins are the result of two binary fissions of a single fertilized egg [83] that develops normally up to the hatching blastocyst stage and then remains in diapause for 7 months; after that the epiblast undergoes two binary fissions, resulting in four conceptuses [84, 85]. Other vertebrate models show an association of a delayed embryo development with the insurgence of monozygotic twins. In particular, artificial modulation of oocyte and embryo development in vitro such as decreasing incubation temperature in fish [86], reducing oxygen availability in mice [87], or delaying ovulation in rabbit [88] may induce a rise in the insurgence of monozygotic twins. Moreover, an artificially induced mechanical split manipulation of the preimplantation embryo at different developmental stages has been associated with the birth of monozygotic twins in cattle (blastocyst) [89], pig (morula and blastocyst) [90], and rhesus monkey (2-cell, 4-cell, and blastocyst) [91].

In humans, insurgence of monozygotic twins has been attributed to hormonal ovulation induction (natural 0.45% vs. ART 1.2%) [92], natural or induced modification(s) in the architecture of the ZP (perforation upon ICSI, assisted hatching) [93–95], and extended in vitro culture conditions [96, 97]. The mouse model shows a comparable incidence of monozygotic twinning (1–1.75%) [98, 99], and their embryos

make a useful proxy for the investigation of the possible causes of monozygotic twinning in assisted reproduction.

There have been two case reports showing human blastocysts herniating from small breaches in the ZP generated upon sperm injection [94, 95], resulting with a so-defined 8-shaped hatching (representative schematic and picture in Fig. 46.1c, d), which resulted in double ICM-like masses in two blastocoels [100, 101], one of which yielded a monozygotic twin pregnancy [100]. The "herniation" hypothesis was also supported by a recent study performed in the mouse [102], where it was shown that extended in vitro culture led to a high frequency of "8-shaped" hatching patterns in both embryos conceived in vivo and in vitro; this hatching pattern leads to an increased occurrence of split ICMs, positive for OCT4 expression, a transcription factor that is exclusively expressed in the ICM cells in the embryo and that maintains ICM cells' pluripotency [103].

While it has been shown that mechanically induced splitting of the mouse morula [104], 2–8-cell stage human embryos [105], and mouse blastocysts [102] results in monozygotic twinning, other factors such as culture media/conditions might cause the embryo to be more likely subjected to splitting in vitro upon mechanical insult, perhaps through induced modification of their epigenome [106]. For example, we occasionally observe zona-free embryos developing into two separate blastocysts (Fig. 46.2a). In this instance, no herniation induced by the ZP could be inferred (as indeed, no ZP was present). These findings are consistent with previous studies showing that the complete removal of the ZP prior to blastocyst transfer does not reduce the incidence of monozygotic twin pregnancies [107]. Because culture media have been shown to induce epigenetic alterations in mouse and human embryos [108–110], one could hypothesize that culture conditions might promote the embryos to "split" at some point during development and perhaps sometimes develop two ICMs, one more visible plus an extra cryptic one, made of few cells and not clearly visible under the microscope, which could continue dividing and eventually be the basis for monozygotic twins (Fig. 46.1e, f). If this hypothesis is correct, we could envision experiments using embryos from transgenic mutant mice with constitutive epigenetic alterations at defined genetic loci (alterations normally induced under in vitro

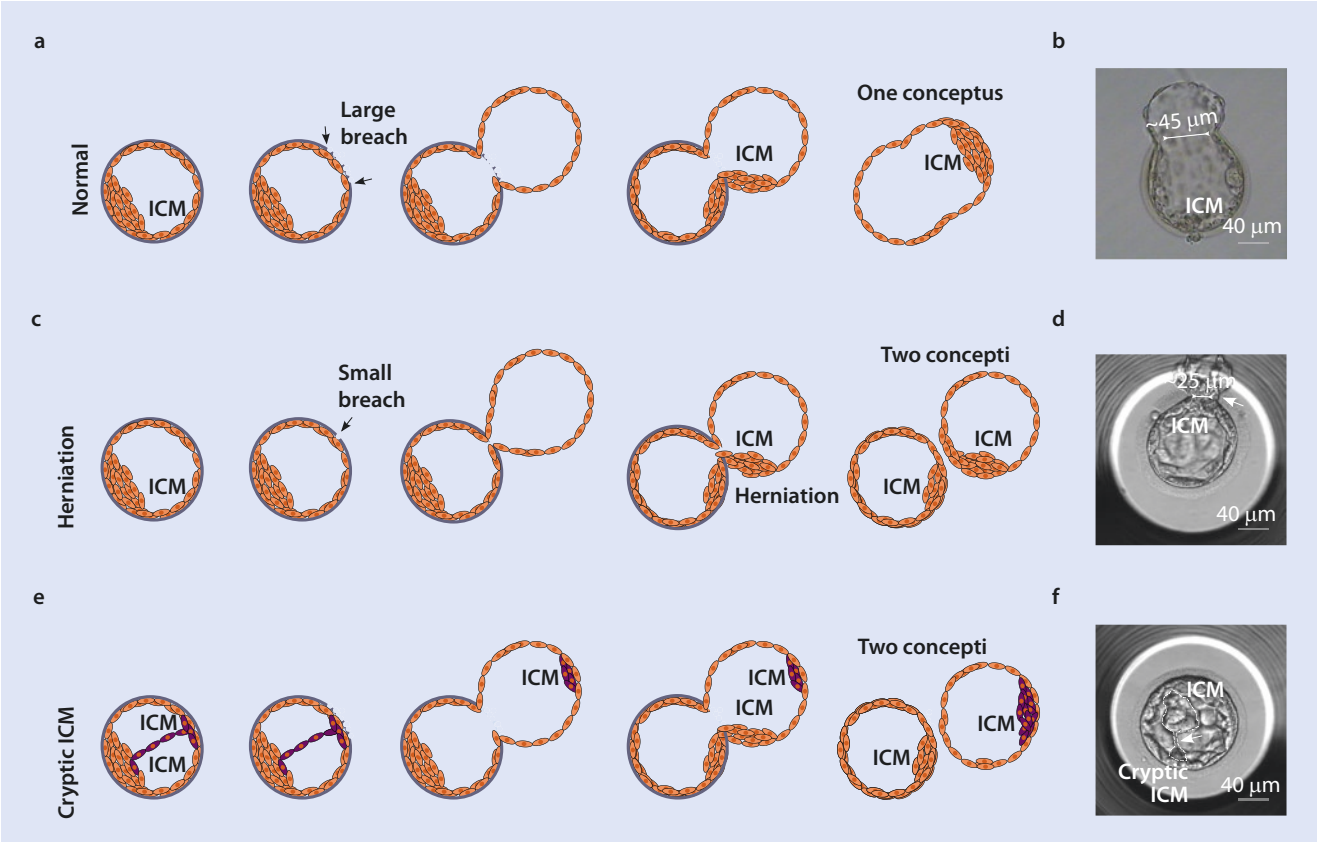

Fig. 46.1 Possible outcome upon laser-assisted hatching. **a** Hatching blastocyst escapes from the manipulated ZP, encountering no obstacles. **b** Representative picture of a hatched blastocyst which successfully implanted. The opening generated was ~45 µm. **c** Small breaches in the ZP may lead to monozygotic twins. Blastocyst escaping through a small (~25 µm) breach in the ZP may undergo ICM herniation and split, with the generation of two genetically identical blastocysts. **d** Representative picture of an "8-shaped" hatching blastocyst. Picture from time-lapse video recording (Embryoscope, VitroLife). Arrowhead shows the herniation of the ICM during embryonic escape from the ZP. **e** ICM bridges. During the completion of cavitation, ICM bridges are occasionally observed. This event could be associated with the presence of a "cryptic" ICM which could eventually result in the development of a second conceptus. This event has been observed to raise the question independently of the AH procedure. **f** Representative picture of an ICM bridge and a possible cryptic ICM

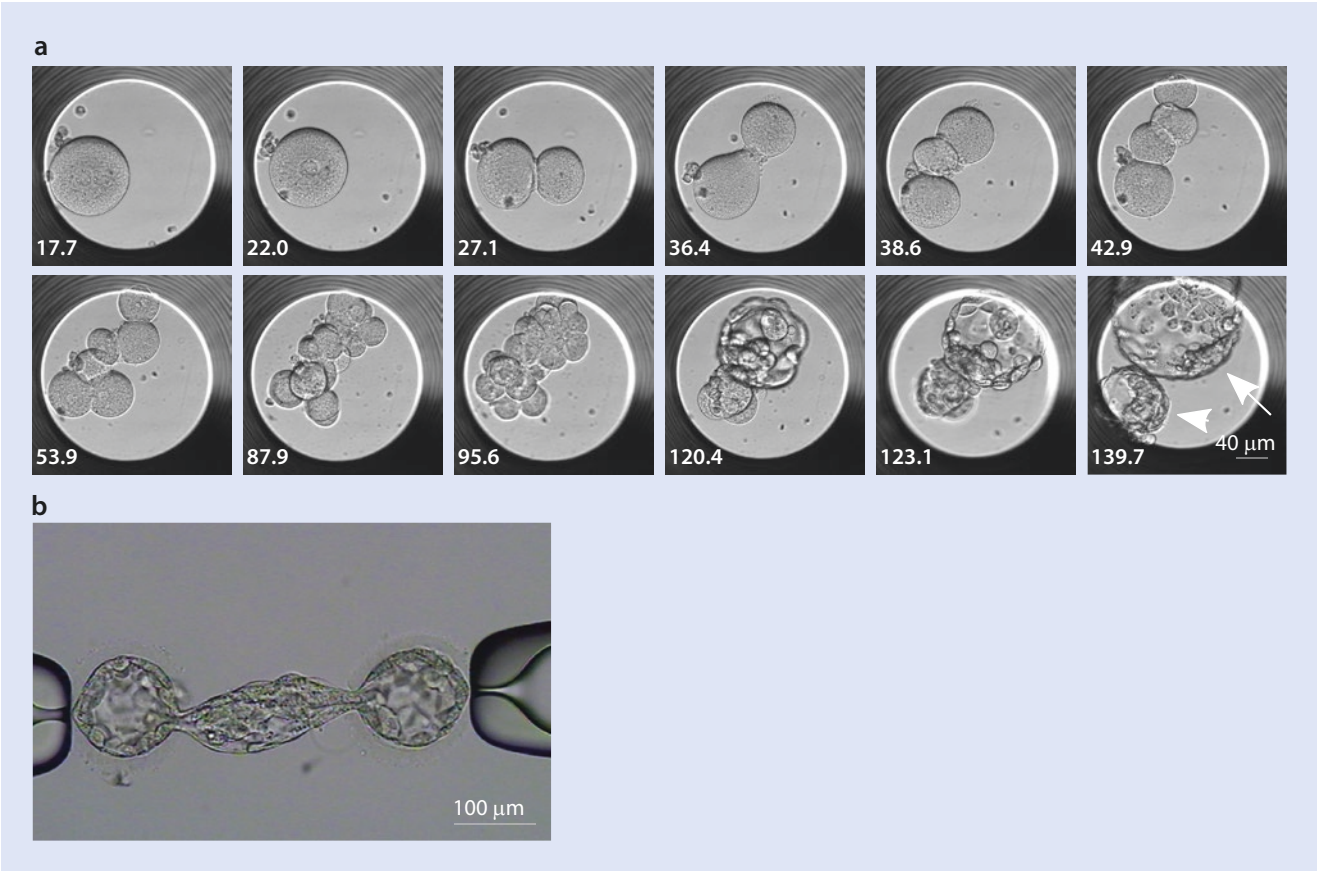

Fig. 46.2 Cases from our fertility treatment programs. **a** Time-lapse imaging frames show an embryo without ZP dividing and splitting into two embryos which ultimately proceed through compaction and cavitation independently and form two disjoint blastocysts (white arrow and arrowhead) which were cryopreserved 5 and 6 days post insemination. As of the date of the drafting of the present manuscript, these two blastocysts were not transferred as the patient became pregnant with a third single blastocyst transfer. Captions are hour post insemination. **b** Embryos cocultured and fusion: hatching embryos at the 8-cell stage that are cocultured may potentially lead to blastocyst fusion as they hatch from their separate ZPs. Although feasible using laser technology, at this stage it might be challenging to divide the blastocysts while preserving their viability. Ongoing studies at our practice are performed to prevent this reoccurring phenomenon

culture conditions) that should activate or silence signaling pathways that may lead to the development of this second "cryptic" ICM and consequentially a higher incidence of monozygotic twins in their litters.

46.4 Current Methodologies for Assisted Hatching

Implantation requires embryo-endometrium contact as the blastocyst hatches out of ZP in the uterus following embryo transfer. Failure of the embryo to escape the ZP is believed to be related to low implantation rates for poor prognosis patients and the main goal of AH is indeed to overcome this obstacle [111]. Here, we describe protocols for the different AH methodologies reviewed above, which include mechanical (partial zona dissection), chemical (zona drilling), and laser AH techniques.

Traditional AH treatments, including mechanical and chemical AH, were mostly performed at the cleavage stage (day 2/3) after fertilization prior to embryo transfer and blastomere biopsy, while the newer laser AH technology is routinely performed prior to blastocyst transfers and trophectoderm biopsies [111]. Mechanical AH or PZD involves making a small slit opening in the ZP using a simple microneedle. A holding pipette is used to hold the embryo in place at 9 o'clock, with the largest perivitelline space at 12 o'clock. The needle is then pierced through the ZP into the perivitelline space at 1 or 2 o'clock position and out the other side of the zona at 10 or 11 o'clock position. The holding pipette suction is released, and the embryo is held by the needle. The holding pipette is rubbed against the pierced area of the zona until it is completely opened in a slit-like fashion [49, 112]. An improvement of this technique is the three-dimensional PZD, where a second slit is made in the ZP at a right angle to the first cut, leaving a cross-shaped hole. This allows a larger opening while still protecting the embryo during transfer [44, 49].

Chemical AH treatment uses acidic Tyrode's solution to create a hole in the ZP. An AH dish is prepared using aseptic techniques, with a large 30–60 µl drop of medium supplemented with 10% protein in the center of the dish. Using a clean pipette, a ~10 µl drop of Tyrode's solution is placed

above the medium drop, and both drops are overlaid with mineral oil. Micro-tool setup includes a holding and hatching pipette correctly positioned and aligned on the micromanipulator. The embryos selected for AH are moved into the hatching dish and placed in the medium drop. The dish is placed on the heated stage of the microscope, and the embryo placed in the center of the view field at 40× magnification. The holding pipette is lowered into the drop and suction is applied to hold the embryo at the 9 o'clock position on the bottom of the dish. The hatching pipette is preloaded with the acidic Tyrode's solution, and is brought close to the ZP in an area near the perivitelline space and away from any blastomeres. Holding pipette, hatching pipette, and embryo should all be in focus in the field of view, with ≤5 μm space between the opening of the hatching pipette and the *zona* surface. The solution is then gently expelled against a small area of the ZP until a hole is made (~30 μm), which should take no more than 10 s. The hatching needle is gently moved up and down to ensure the acid is dispersed over a section of the ZP. Once the desired hole is achieved, suction is applied by the hatching pipette to prevent any acid from entering the embryo. The embryo is then immediately rinsed off several times and placed back in culture [44, 66].

Laser AH has grown in popularity since the 1990s with its advanced technology and ease of use in the IVF laboratory. This technique uses a noncontact 1.48 μm diode laser beam and computer software to breach the ZP in a controlled manner. The laser technology allows for easier AH of both day 3 embryos and fully expanded blastocysts for transfer. Selected embryos need to be artificially shrunk down away from the ZP prior to AH to "allow space" for the laser. The target on the laser software is moved over the selected portion of the zona, placing it in the path of the laser beam. Repeated pulses of the laser using a computer mouse or footpad produce the desired size of *zona* breaching [111].

A laser AH protocol for day 3 embryos requires only an inverted microscope outfitted with a 1.48 μm diode (Hamilton Thorne-Zilos) laser and software (Fig. 46.3a). The selected embryos are moved to a separate well in the culture dish and placed on the heated stage. The desired location for AH is selected based on the largest perivilline (PV) space away from any blastomeres (Fig. 46.3b). The embryo is then positioned in the middle of the microscope field of view on the laser-grade objective lens displaying the laser target. With the laser power set at 300 mW and 260 μs pulse duration, the target is placed over the outside edge of the ZP, and the laser is applied to create the first hole (Fig. 46.3b). Using the stage manipulator, the target is positioned immediately next to the first hole and the laser is applied again. This is repeated until the PV space is reached. Using this procedure, a breach of ~40–45 μm diameter should be opened to later avoid ICM herniation (Fig. 46.1a, b). Once all selected embryos have been hatched, the dish is placed back into the incubator. Under these conditions, when we culture ≥2 embryos in the same dish, we occasionally have observed two blastocysts attempting to merge (Fig. 46.2b). At this point, it is challenging to separate the two blastocysts, and this condition may lead to the discard of both embryos.

To prevent this issue, we also perform AH at the blastocyst stage. As per our protocols, laser AH of expanded blastocyst requires a pre-made 0.2 M sucrose solution to artificially collapse the embryo (Fig. 46.3c). A ~50 μl drop is placed in the lid of a Falcon® dish, and overlaid with mineral oil. The drop is aspirated and refreshed with fresh solution prior to hatching to prevent evaporation. The blastocysts are transferred into the sucrose drop, and the drop is drawn down to place the embryos on the bottom of the dish. The dish is then placed on the heated stage of the inverted microscope with the laser. Once the embryos have collapsed away from the ZP, the stage manipulator is used to reposition the target, and the laser is fired in a straight line to cut off a

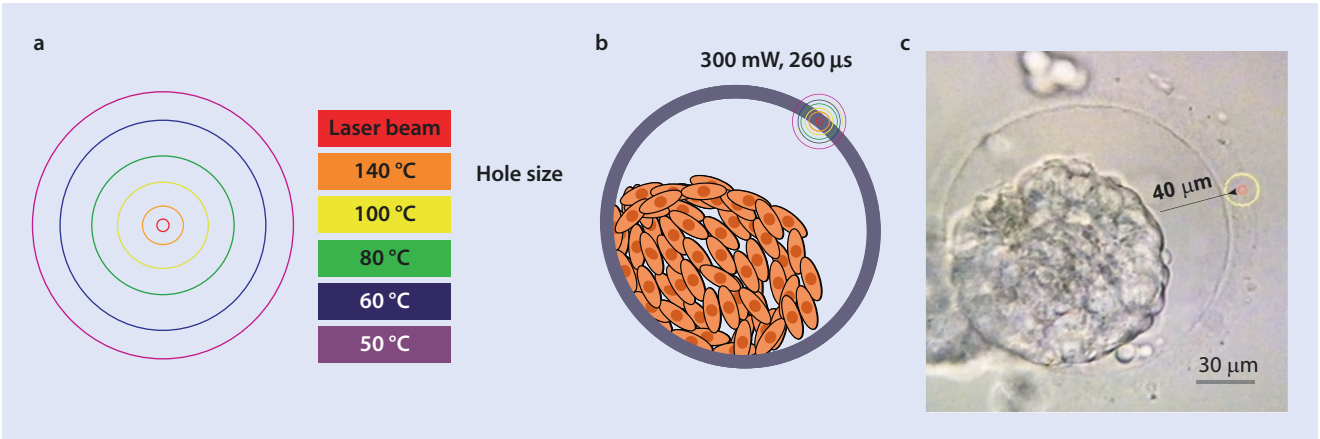

Fig. 46.3 Laser-assisted hatching. **a** Schematic of predicted thermal profile of the Hamilton Thorne Isotherm Rings™ which shows the temperature that the tissue and cells around the center hole (orange) may potentially be exposed to. **b** Schematic shows the conditions we have adopted for laser AH. A laser power of 300 mW and pulse duration of 260 μs help protect a collapsed embryo from the exposure of laser-generated heat. **c** We estimate that maintaining the blastomeres/trophectoderm ~40 μm away from the center hole of the laser prevents the embryo from exposure to the laser-induced heat, which guarantees minimal to no physiological stress to the embryo and consequently no impact on survival and implantation potential

Table 46.1 Cycle outcomes from transfer of non-hatched day 3 embryos (2010–2015)

	Total	Not pregnant	Biochem.	Ectopic/heterotopic	CIG	CIG (%)
Total (2010–2015)	2420	1213	164	20	1023	42.27
<30	277	123	21	6	127	45.85
30–32	521	261	26	3	231	44.34
33–34	509	248	36	4	221	43.42
35–37	834	425	57	6	346	41.49
>37	279	156	24	1	98	35.13

Pregnancy outcomes from patients clustered by age from 2010 to 2015. *CIG* clinical intrauterine gestation

portion of the ZP. After this process is completed, the embryos are rinsed 2–3 times before being placed into their culture dish.

As clinical tools, all of the AH procedures have their benefits and drawbacks. It is crucially important to minimize the time the embryos are exposed to atmospheric air during the procedure. The goal of AH is to generate a breach in the ZP in an efficient manner while keeping proper pH and temperature [44]. The more traditional techniques of PZD and zona drilling require extensive technical skills to achieve consistent results. PZD can be performed relatively quickly but results in inconsistent sized holes in the ZP. Indeed, small breaches in the ZP can lead to entrapment and herniation of the developing blastocyst, possibly increasing the chances of monozygotic twinning in the case where the ICM is pinched in two. On the other hand, large breaches can result in blastomere expulsion during the transfer [44, 111, 113]. *Zona* drilling using acidic Tyrode's solution creates larger and more consistent holes in the ZP, but involves possible exposure of the embryo to the acidic solution and must be performed quickly. Each embryo must be quickly and extensively rinsed to avoid unnecessary exposure of the cells to acidic medium, which increases the time needed to perform this technique [44, 111]. Laser AH requires a microscope equipped with a laser system, and lately, most laser systems have been designed to be easily adaptable to existing inverted microscopes. Laser AH is highly efficient, is technically easy to use, generates consistent results, and has no apparent negative impact on the embryo developmental performances [44, 49, 111, 114, 115].

46.5 Assisted Hatching on Fresh and Frozen Embryo Transfers from Poor Prognosis Patients: Current Data from Shady Grove Fertility Clinical Programs

We extensively investigate the effect of AH on patients clustered by age, over the period 2010–2015 in our fertility clinics, and here we report tables summarizing the data (Tables 46.1, 46.2, 46.3, 46.4). A general overview would suggest that AH might negatively affect implantation rate and pregnancy outcome (Table 46.3) when compared with embryos to which AH was not applied (Table 46.1). However, AH is routinely used with "poor" prognosis cases: a few examples are embryos that present a "poor" developmental pattern, embryos that failed to be fertilized using conventional insemination and are re-inseminated using ICSI, embryos from patients with recurrent failure of infertility treatments, and embryos from oocytes of 38 years of age or above. These studies present a number of limitations that may affect the validity of the study outcome [68]. For example, to assess the effect of AH on any embryo, it would be necessary to include as control sibling embryos with a comparable developmental pattern to which AH was not applied.

One more instance in which we routinely adopt AH is when cryopreserved embryos are being transferred. Table 46.5 summarizes the fertility outcomes from our fresh and frozen embryo transfer (FET) cycles over the period 2010–2015.

Table 46.2 Pregnancy outcomes from transfer of non-hatched day 3 embryos (2010–2015)

	SAB	THA	Still.	Unknown	Delivered	Delivered (%)
Total (2010–2015)	160	5	9	2	847	82.8
<30	8	1	3	0	115	90.55
30–32	29	1	1	0	200	86.58
33–34	35	1	1	0	184	83.26
35–37	62	1	3	2	278	80.35
>37	26	1	1	0	70	71.43

Pregnancy outcomes form patients clustered by age from 2010 to 2015. *SAB* spontaneous abortion, *THA* therapeutic abortion, *Still.* still birth

Table 46.3 Cycle outcomes from transfer of hatched day 3 embryos (2010–2015)

	Total	Not pregnant	Biochem.	Ectopic/heterotopic	CIG	CIG (%)
Total (2010–2015)	4144	2778	245	19	1102	26.59
<30	60	41	3	0	16	27.67
30–32	193	127	10	1	55	28.5
33–34	286	175	22	1	65	22.73
35–37	456	276	29	4	147	32.24
>37	3241	2221	188	13	819	25.27

Pregnancy outcomes from patients clustered by age from 2010 to 2015. *CIG* clinical intrauterine gestation

Table 46.4 Pregnancy outcomes from transfer of hatched day 3 embryos (2010–2015)

	SAB	THA	Still.	Unknown	Delivered	Delivered (%)
Total (2010–2015)	306	21	1	3	771	69.96
<30	1	0	0	0	15	93.75
30–32	7	1	0	0	47	85.45
33–34	14	0	1	0	50	76.92
35–37	27	0	0	0	120	81.63
>37	257	20	0	3	539	65.81

Pregnancy outcomes from patients clustered by age from 2010 to 2015. *SAB* spontaneous abortion, *THA* therapeutic abortion, *Still.* still birth

Table 46.5 Fertility outcome of laser-assisted hatched day 5 and day 6 blastocysts from "poor prognosis" patients upon fresh or frozen transfers (2010–2015)

	Total	Not pregnant (%)	Biochem. (%)	Ectopic/hetero-topic (%)	CIG (%)
Fresh	1679	45.74	9.53	0.54	44.19
Frozen	989	40.24	10.62	0.81	48.33

Pregnancy outcomes from "poor prognosis" patients ≥38 years of age, from 2010 to 2015. The current trend from our data seems to indicate better implantation rate when frozen cycle is performed. *CIG* clinical intrauterine gestation

While we find it hard to select appropriate controls in our clinical settings, we have found no detrimental effect of AH using our protocols. Based on the data collected over the past few years (Table 46.5) and in the light of data from previous reports showing that AH is ordinarily applied to embryos that have likely less chances to successfully implant [68], we adopt AH as a precautionary technique to assist embryos to escape from the ZP before implantation.

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Review Questions

1. What is the most recent general understanding on the mechanisms of fertilization, zona hardening, and embryo hatching?
2. How many assisted hatching procedures are being currently adopted and which is the most effective and less-invasive approach?
3. Does assisted hatching improve implantation and fertility outcome in all clinical cases or just in specific cases?

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Biopsy Procedures on Oocytes and Embryos

Contents

Polar Body Biopsy

Markus Montag

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Learning Objectives

- What kind of equipment is required for polar body biopsy?
- When is the best time to perform polar body biopsy?
- How is zona opening best accomplished?
- How is the biopsy procedure performed?
- What are potential pitfalls during biopsy?
- How are polar bodies harvested for later analysis?
- What is the current status of polar body biopsy?

The chapter closes with questions that review the major aspects of the learning objectives.

47.1 Introduction

Polar body biopsy was first introduced in 1990 by Verlinsky and collaborators [1]. Since then, several groups have applied polar body biopsy to a variety of diagnostic applications such as detection of single gene disorders [2–4], translocation analysis [5], HLA typing [6] and detection of X-linked disorders [7]. However, to date, most cases of polar body diagnosis are performed for aneuploidy screening [8–11].

Polar bodies are by-products of the meiotic division. Removal of the first and/or second polar body is an indirect approach allowing the genetic status of the oocyte to be inferred from that of the polar body. The first polar body is not required for successful fertilization or normal embryonic development. The second polar body, which is extruded from the oocyte after initiation of the fertilization cascade by the spermatozoa, is similarly not required for subsequent embryo development, although it does persist up to the blastocyst stage [12]. Therefore, removal of both polar bodies for the purposes of genetic diagnosis is considered having no deleterious effect on the developing embryo.

47.2 Technical Aspects of Polar Body Biopsy

The most relevant parts of polar body biopsy are the timing of biopsy, the atraumatic opening of the zona pellucida and the removal of the polar bodies.

47.3 Equipment for Polar Body Biopsy

Like all other manipulation procedures, polar body biopsy requires an inverted microscope with a heated plate and micromanipulators. Based on the current practice, a non-contact near-infrared diode laser system should be part of the setup for opening of the zona pellucida prior to biopsy [13]. A holding capillary – similar to that used for intracytoplasmic sperm injection (ICSI) – and a bevelled and flame-polished capillary for polar body biopsy with an inner diameter of approximately 18 µm are required for the actual manipulation and biopsy of the polar bodies. A stereomicroscope, dishes, pipettes and other labware are part of the standard laboratory setup; however, a special pipette may be needed for transfer of polar bodies into reaction tubes.

47.4 Timing

An oocyte presenting a first polar body is usually considered to be in metaphase II. However, recent investigations using polarization microscopy have shown that some oocytes may be still in telophase I due to the presence of a connective spindle strand between the first polar body and the oocyte [14] (Fig. 47.1). Such a spindle bridge is a remnant of the meiotic division and is only present for a limited time period of 1–2 h after extrusion of either the first or the

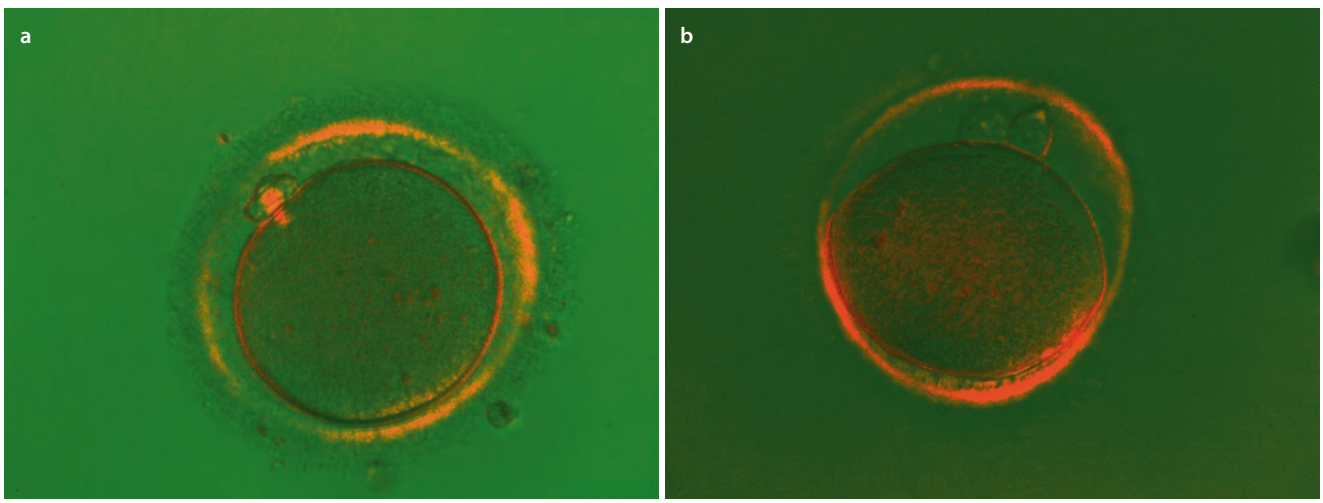

Fig. 47.1 **a**, **b**: For an optimized biopsy of the first and/or second polar body, the presence of a connective spindle bridge between one of the polar bodies and the oocytes can be visualized by polarization microscopy. If a spindle is present **a** the biopsy should be performed 1.5–2 h later, as the remnants of the spindle still may connect to chromosomal material, which imposes the risk of complete or partial removal of DNA during biopsy. The absence of a spindle **b** denotes that polar bodies are no longer connected by spindle strands and can be removed with such a risk

second polar body. Therefore, it is important not to biopsy polar bodies within a too short time period after their formation, because chromosomal material from the oocyte may still be attached to these spindle fibres and pulled out during biopsy. For the first polar body, this does usually only apply if one considers performing the biopsy prior to or immediately after the ICSI procedure – unless one can control for the presence and location of the spindle by using a polarization microscope. The risk is higher for the second polar body, which usually is extruded within 1.5–3 h after ICSI [15].

In view of this, several biopsy strategies have been discussed for polar body biopsy [13]. Removal of the first and second polar bodies can be done at separate time points or at the same time point. The sequential approach has the clear advantage that one can always distinguish the first and second polar bodies – which is of uttermost importance for diagnosis of monogenetic diseases. However, care has to be taken to remove both polar bodies through the same opening (see Pitfalls of polar body biopsy).

Simultaneous biopsy of the first and second polar bodies requires only one manipulation and helps reduce stress to the oocyte and is best accomplished in a time window of 8–14 h after fertilization. Too early biopsy bears the risk of spindle remnants in the second polar body, and too late biopsy may result in a first polar body that already started disintegration or degeneration. The latter problem is especially important if the analysis is based on fluorescence in situ hybridization (FISH) as it may contribute to diagnostic failures [8].

Regarding isolation of polar bodies for aCGH, the timing of biopsy of the second polar body seems to influence the amplification results. It was reported that too early biopsy of the second polar body (4–6 h post ICSI) may slightly lower the amplification efficiency, and this effect disappeared after adjustment to later biopsy times (>8 h post ICSI) [16].

Independent of whether a sequential or simultaneous approach has been chosen, it is mandatory to biopsy and to analyse both polar bodies. It has been shown in younger women that the first polar body is more prone to meiotic errors causing aneuploidies than the second polar body, whereas the opposite holds true in older women [17]. Hence, one could be tempted to conclude for chromosomal screening that analysis of the first polar body is more important in younger women and of the second polar body in older women. However, a reliable diagnosis can only be based on the analysis of both polar bodies, and results from clinical studies on the concordance of PB 1 and 2 analysis and the corresponding oocyte gave direct proof to this [18].

47.5 Zona Opening

Various methods have been proposed for the opening of the zona pellucida and subsequent removal of polar bodies: chemical, mechanical and laser-assisted opening.

47.6 Chemical Opening

Acidic Tyrode's solution was the first method ever used for opening of the zona pellucida by chemical means [19]. Although acidic tyrode can be applied at the embryo stage, there was an inhibitory effect on embryonic development when oocytes were exposed to acid tyrodes [20]. Therefore, since both the oocyte and polar body are sensitive to the effects of acid, zona drilling by acidic Tyrode's solution is unsuitable for polar body biopsy.

47.7 Mechanical Opening

A very efficient mechanical technique was elaborated by Cieslak et al. [21] and is based on three-dimensional zona dissection and subsequent biopsy. For this procedure, the oocyte is affixed to the holding capillary. Using a sharp needle, a slit is made close to the area where the polar bodies are located. After turning the oocytes by 90°, a second slit is made by creating a cross-like incision in the zona, which allows accessing the polar bodies. This method can be performed with simple glass tools; however, multiple steps including dissection, release and rotation of the oocyte are needed. Therefore, this procedure is technically difficult and requires extensive experience.

Another approach is the use of a bevelled micropipette (12–15 µm in diameter), which due to its sharpness assists in opening the zona. For this technique, the oocyte is oriented so that the polar body is located at the 12 o'clock position. The bevelled micropipette is passed through the zona and into the perivitelline space tangentially toward the polar body, which can then be aspirated into the pipette. This method works very well if only one polar body is biopsied; however, it is more tedious if the first and second polar bodies need to be biopsied and if both are not lying close to each other. Naturally, this method bears a certain risk of damaging the oocyte due to the sharpness of the needle.

47.8 Laser-Assisted Opening

The ultimate way of opening the zona pellucida is by a laser beam. Lasers were initially used to assist fertilization in cases of severe male factor infertility. The introduction of laser-assisted zona opening [22] has entered the field of polar body [11, 23] and embryo biopsy [24] and has helped in reducing the rate of biopsy damages as well as the time required [25]. In a recent expert survey among embryologists, laser-assisted zona opening was considered to be the only methodology that should be applied for polar body biopsy [13] as it offers a very fast access to the perivitelline space and allows to perform the required manipulations within a minimum of time.

47.9 Biopsy Procedure

The whole procedure of simultaneous laser-assisted polar body biopsy is illustrated in Fig. 47.2. Laser-assisted polar body biopsy is best accomplished when the oocyte is affixed

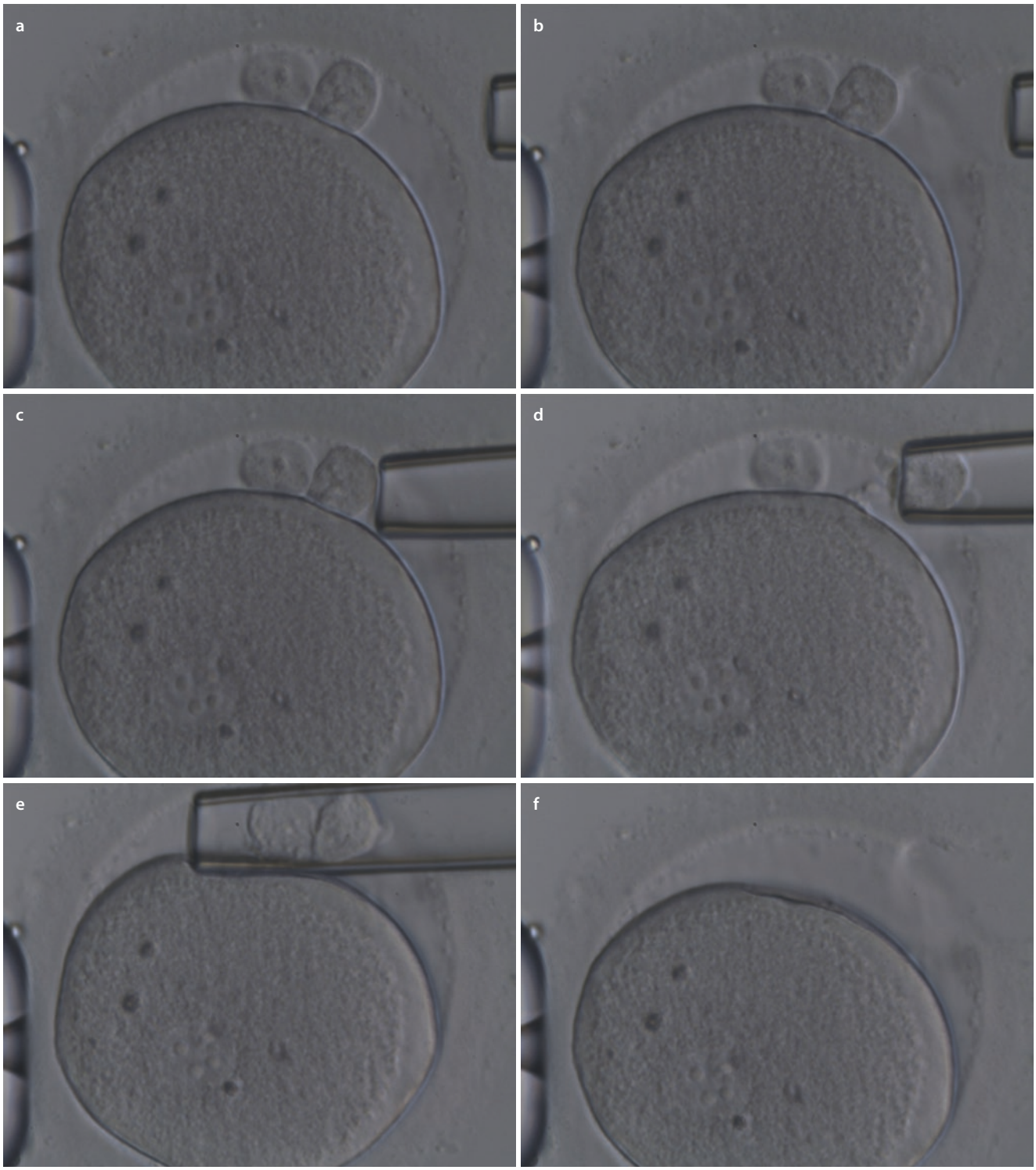

Fig. 47.2 **a**–**g**: Polar body biopsy following polarization microscopy For biopsy, the first and second polar bodies were aligned with a holding capillary so that the second PB faced to the biopsy capillary **a**. Using a non-contact 1.48 µm diode laser, an opening was introduced into the zona pellucida using 2–3 laser shots **b** through which the biopsy capillary could be easily introduced **c**. The second polar body is usually connected to the oolemma via a cytoplasmic strand **d**. In order to remove the second polar body without damaging the oocyte, it is *not* recommended to suck the second PB into the capillary, as shown in **d**. Instead, the capillary is pushed slowly over the second PB and toward the first PB **e**. Once the first PB enters the capillary, the strand between the second PB and the oolemma breaks due to shear stress, and both polar bodies can be easily removed **e**, leaving the oocyte without any damage **f**. Polar bodies should be placed in one droplet for further processing for FISH analysis **g** or in two different droplets if a PCR-based analysis is performed

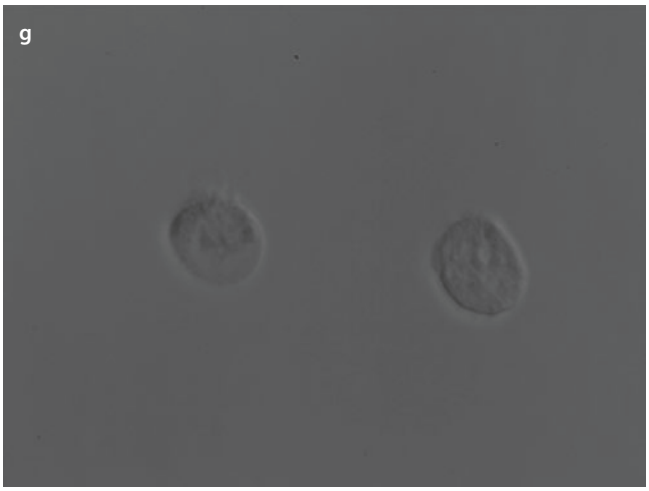

Fig. 47.2 (continued)

to the holding capillary with the first polar body at the 12 o'clock position and the second polar body located right of the first one but in the same focal plane. An opening of 18–25 µm is drilled at 2–3 o'clock, and by pushing the biopsy capillary into the perivitelline space, both polar bodies can be removed simultaneously. The positioning of the second polar body next to the aspiration capillary allows pushing the second polar body far to the left side toward the holding capillary. This stretching movement usually is sufficient to break the cytoplasmic bridge between the second polar body and the oocyte. Due to the use of a blunt-ended capillary, even manipulation in direct vicinity to the oolemma does not damage the oocyte.

In general, the size of the drilled opening is usually in the range of 20 µm or less, but it can be easily adjusted to the diameter of the aspiration capillary. As the capillary can be introduced through the laser-drilled opening, there is no need for a sharp aspiration needle. This allows the use of flame-polished, blunt-ended aspiration needles and greatly reduces the risk of damaging the polar body or the remaining oocyte. The procedure is accurate, reproducible and safe, and it also reduces the number of cells that cannot be reliably diagnosed as a result of technical problems during the biopsy procedure [26]. Another benefit is that laser drilling and subsequent biopsy can be performed without changing the capillaries in contrast to mechanical zona drilling where one either needs a double capillary holder for the drilling and the polar body biopsy capillary or another setup for subsequent biopsy.

47.10 Pitfalls of Polar Body Biopsy

The different approaches to generate an opening in the zona pellucida do result in different characteristics of the shape of the openings as well as the behaviour of the corresponding oocytes and embryos. To date only the effect of laser drilling has been studied intensively.

Although the use of laser seems to be easy and straightforward, it is still essential that the technique is trained properly in order to avoid possible pitfalls [27]. Laser opening of the zona can be performed at a very high precision, giving reproducible results. Consequently, it was shown that laser-assisted biopsy does not interfere with further development of mouse embryos [23] as long as the laser is used in a proper way [27], and a few examples will be given below.

Laser-drilled openings stay permanently in the zona, and therefore gentle handling during subsequent transfer of oocytes to other media droplets and even during the embryo transfer is strongly recommended.

Depending on the size and position of the laser openings, inappropriate hatching may occur at the blastocyst stage [27]. If the biopsy of both polar bodies is done in a sequential approach at different time points, one should avoid drilling another opening. If polar bodies were retrieved through separate openings, problems may arise at the time of hatching because the embryo could hatch through both openings simultaneously and therefore may get trapped within the zona [27] and either split or get stuck and degenerate.

While introducing an opening in the zona, care should be taken to generate a sufficiently large opening which allows consecutive hatching at the blastocyst stage, because smaller openings (<15 µm) may also cause trapping of the embryo followed by splitting or degeneration [27].

Independent of the method used for biopsy, it is extremely important to note the shape of the polar body. Especially, a fragmented polar body should be classified as that because this does require special care during later transfer for further evaluation. If fragments are lost, one will get an incomplete diagnosis or even a misdiagnosis.

47.11 Preparation of Polar Bodies for Subsequent Analysis

Once polar bodies are biopsied, they need to be transferred for further analysis by FISH or amplification of the DNA for subsequent analysis by aCGH, NGS or SNP array. This transfer is a crucial step as it bears the risk of loss of material or even of contamination.

47.12 Transfer for FISH

Immediately after biopsy of an oocyte, the corresponding polar bodies are placed in a neighbouring droplet of medium until all oocytes are biopsied. For FISH, it is not essential to place the first and second polar bodies in different droplets, as they can be visually distinguished during fluorescence evaluation. Due to the small cytoplasmic content of the polar bodies, a special pretreatment such as hypo-osmotic swelling or proteinase/pronase treatment prior to FISH is

not necessary. For transfer onto the glass slide, polar bodies of one oocyte are removed from their drop and transferred into a tiny drop (0.2 µl) of water placed on a clean glass slide. The small volume guarantees that the polar body attaches to the slide within a small area and that the fluid dries out very fast, which reduces the risk of a dislocation of the polar body on the slide. It is recommended to use for this transfer the biopsy capillary and to perform the complete procedure under visual control at the microscope. Placing the polar bodies directly at the bottom of the slide prevents floating and rupture of the polar bodies. The drying process should be observed under a stereomicroscope, and the final location of the polar body after air-drying should be marked on top of the slide by encircling with a diamond marker. With some experience, polar bodies from 6 to 10 oocytes can be placed within a round area of 10 mm, each encircled with a diamond marker. Subsequent fixation can be performed with 2–3 drops of 10 µl methanol:acetic acid (3:1, ice-cold −20 °C) followed by another fixation after air-drying using methanol at room temperature for 5 min [28].

47.13 Transfer for PCR

In contrast to the isolation for FISH, the differentiation of the first and second polar bodies is crucial for any PCR-based evaluation, either for monogenetic diseases or for screening. Therefore, the first and second polar bodies are released after biopsy in different droplets with medium in a dish covered with mineral oil. This facilitates the subsequent transfer of both polar bodies into separate reaction tubes. Using a high-contrast stereomicroscope, polar bodies can be easily identified in the medium droplets and aspirated for immediate transfer by using an unbreakable plastic capillary with a fine tip, similar to those used for oocyte denudation, which has a diameter ranging between 80 and 140 µm. If a standard low-volume pipette (0.2–2.0 µl) is used, care should be taken to use droplets for polar body sampling of at least 10 µl of volume as this facilitates to aspirate the polar bodies without sucking up any mineral oil.

It is important to note that the subsequent DNA amplification procedure, which is performed in the reaction tubes, requires a well-defined volume and a special medium base (e.g. phosphate-buffered saline, PBS). Therefore, polar bodies should be transferred with a minimal carryover of the sampling medium (which is usually HEPES- or MOPS-based culture medium). A standard setting is to pre-fill reaction tubes with 2.1–2.3 µl of PBS and to transfer polar bodies with 0.4–0.2 µl of medium into this solution to give a final maximum volume of 2.5 µl. Following the transfer into the reaction tube, it is advisable to rinse the capillary in the sampling droplet in order to verify that the polar body has been placed in the reaction tube, as this process cannot be directly visualized due to the plastic material of the tubes.

47.14 Current Status of Polar Body Biopsy

FISH technology is nowadays less applied for diagnosis in PGS programs but may still be found in combination with polar body biopsy. A major problem of such an approach is the interpretation of FISH results, especially for the first polar body. It is unknown at what time the first polar body has been extruded during the final maturation of the oocyte. Therefore, the chromatin of the first polar body is more prone to an ageing process compared to the second polar body, whose formation and ageing can be precisely controlled as a result of the time point of injection. Ageing affects the quality of the DNA, the coherence of the chromatids, its dispersion after isolation on a slide and hence hybridization efficiency. In contrast, isolation and amplification of DNA from the first (and second) polar body for PCR-based diagnostic methods can be accomplished with over 90% success rates even from polar bodies that show signs of degeneration.

Independent of the diagnostic approach, the use of polar bodies only allows a diagnosis of the maternal contribution, whereas the paternal factor remains inaccessible.

Despite these shortcomings, polar body biopsy has been discussed in recent years as a potential alternative to blastomere biopsy on day 3 [29].

Whereas some studies report a high or acceptable correlation for predicting aneuploidy based on polar body and aCGH [18, 30], others question the accuracy of polar body diagnosis due to the high incidence of post-zygotic errors [31, 32]. Another conflicting topic is the occurrence of reciprocal chromosome aneuploidy results in the first and second polar bodies of an oocyte. This was considered as a negative factor, but a recent study showed that this situation mostly gives rise to normal euploid embryos [33], and the birth of a healthy child has been reported from an oocyte with reciprocal aneuploid polar bodies [34]. These findings illustrate the difficulty in the interpretation and diagnosis of polar body-based results.

In combination with comprehensive chromosome screening methods such as aCGH or NGS, polar body biopsy and subsequent diagnosis is the most expensive approach that can be chosen. First, not every oocyte develops into a viable embryo. Even if the diagnosis is postponed to a stage that allows a better judgment of the developmental potential of an oocyte and the corresponding embryo, still both polar bodies from every oocyte should be initially biopsied and sampled. Second, both polar bodies should be separately analysed, which inevitably doubles the costs of the diagnosis.

47.15 Summary

Polar body biopsy is the initial step prior to investigation of the first and second polar bodies regarding genetic dispositions or structural and/or numerical chromosomal disorders. Polar body diagnosis allows concluding on the genetic/chromosomal constitution of the oocyte. The most frequently

used biopsy method is the laser-assisted biopsy followed by mechanical biopsy using zona dissection.

Isolation of polar bodies depends on the methods used for diagnosis. For FISH analysis, both polar bodies can be simply placed in a water droplet on a glass slide, whereas any PCR-based approach requires separate processing of the first and second polar bodies under sterile conditions.

Accurate timing and technique of biopsy are important for optimal results and reduction of oocyte trauma. Differences in techniques may explain differences in the success of polar body diagnosis for PGS reported by different studies. Polar body biopsy requires a proper training in order to achieve good results. However, the need to analyse both polar bodies for genetic as well as for chromosome screening methods in order to get the full picture is a cost-driving factor and a clear disadvantage compared to other methods such as trophectoderm biopsy [35].

Review Questions

1. What kind of equipment is required for polar body biopsy?
2. When is the best time to perform polar body biopsy?
3. How is zona opening best accomplished?
4. How is the biopsy procedure performed?
5. What are potential pitfalls during biopsy?
6. How are polar bodies harvested for later analysis?
7. What is the current status of polar body biopsy?

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Cleavage-Stage Embryo Biopsy

Alan R. Thornhill

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Learning Objectives

- To describe the need for embryo biopsy
- To present the options at what embryonic stages can biopsy be performed
- To review the technical requirements to perform embryo biopsy
- To provide a detailed description on how cleavage-stage embryo biopsy is performed
- To present the applications of cleavage-stage biopsy

The first PGD cycles were carried out in late 1989 in a series of couples at risk of X-linked disease and involved cleavage-stage embryo biopsy [1]. Theoretically, PGD can be accomplished at any developmental stage between the mature oocyte and blastocyst, but to date, only three discrete stages have been proposed: polar body, cleavage-stage, and blastocyst. Clearly, each of these stages is biologically different, and thus, the strategic considerations have both advantages and disadvantages [2] (Table 48.1). However, cleavage-stage biopsy has remained the most widely practiced form of embryo biopsy worldwide (according to the ESHRE PGD Consortium) accounting for approximately 90% of all reported PGD cycles to date [3]. Currently, this embryo biopsy strategy requires the removal of one or more cells from each embryo, making it comparable to amniocentesis or CVS at fetal stages since the primary aim is the removal of sufficient embryonic tissue to allow diagnosis. Cleavage-stage embryo biopsy is a two-step micromanipulation process involving the penetration or removal of part of the zona pellucida surrounding the oocyte or embryo followed by the removal of one or more cells. Many of the biopsy techniques currently in use for human embryos [4] were pioneered in animal models, notably the mouse [5–7]. While the total number of human embryos biopsied in clinical cases is vast, relatively little work has been published to define the relative merits of different biopsy methods and their safety and efficacy in clinical application (Table 48.2). This chapter focuses on cleavage-stage embryo biopsy since the majority of PGD centers and clinical cases reported have employed this technique [3].

Table 48.1 Advantages and disadvantages of cleavage-stage embryo biopsy

Stage	Advantages	Disadvantages
Cleavage-stage accuracy (blastomeres)	Diagnosis of maternally and paternally inherited disease	Chromosomal mosaicism compromises accuracy
	Gender determination possible	Choice of blastomere is critical
	Large body of clinical data available	Time for analysis may be limited
	1–3 cells available for analysis	Most cells in interphase (no karyotypic data)
	Biopsied embryos develop into normal blastocysts	Single-cell-sensitive analysis required
		Reduced embryo implantation potential post-biopsy
2–4 cell	95% embryo cohort available for analysis	Detrimental effects of acid/reduced cell mass
		Possible selected cell allocation to TE/ICM
6–10 cell	1 or 2 cell removal still results in viable development	Reduced embryo cohort on the day of biopsy
		Possible selected cell allocation to TE/ICM

48.1 Penetration of the Zona Pellucida

Until the advent of noncontact lasers for use in micromanipulation (see below), two basic methods were employed for zona pellucida penetration. Both methods were pursued initially as a means to enhance fertilization rates with oligozoospermic men and have now been superseded for this purpose by intracytoplasmic sperm injection (ICSI).

48.1.1 Mechanical Zona Penetration

The first approach, partial zona dissection (PZD), employs a fine needle to penetrate the zona at two separate points around the circumference. The oocyte or embryo is then detached from the holding pipette as it is effectively held on the needle and a gentle rubbing action is made against the side of the holding pipette used to make a slit between the two apertures generated by the needle taking care to avoid damage to the oocyte or embryo [8]. Although a narrow-diameter micropipette can be pushed through such a slit, it is difficult to use one large enough to aspirate cleavage-stage blastomeres, and with the human embryo, pressure on the zona can lead to the lysis of blastomeres and/or, where a slit has been made, force blastomeres out through the slit. The latter approach is used for embryo biopsy in some centers, but requires highly skilled micromanipulation, can be difficult to control, and does not allow precise selection of blastomeres, and the risk of lysis can be high. A modification is to make two slits to create a "flap" or "cross" in the zona that can be flipped open, allowing more flexibility in the size of the opening created. This method is effective for both blastomere and polar body biopsy [9].

Table 48.2 Cleavage-stage embryo biopsy methods—benefits, limitations, and factors critical to success

Zona penetration method	Benefits	Limitations	Factors critical to success
Mechanical	Least invasive to embryo (safer)	Difficult to learn	Operator skill essential
	Improved survival after freeze–thaw?	Operator dependent	Appropriate microtools needed
	Inexpensive	Time-consuming	
Chemical (acidified Tyrode's solution)	Relatively inexpensive	Operator dependent	Acidified Tyrode's solution pH 2.2–2.4
		Difficult to limit aperture size	Sensitive control of acid
	Widespread clinical experience	Effect on cryopreservation?	Rinse acid from embryos
		Double tool holder optimal	
Laser (1.48 µm noncontact diode)	Rapid and reproducible	Capital cost (30–60,000 US dollars)	Laser alignment and calibration
	Simple to use	Not all systems portable	Pulse duration and number
	Documentation/measurement software	Invisible thermal damage/stress	Distance between laser and zona
Cell removal method			
Aspiration	Ability to select cell	Cell lysis during aspiration	Appropriate microtools needed
			Sensitive suction device
Fluid displacement	Aspiration pipette does not contact cells	Limited ability to select cell	Operator skill essential
Mechanical displacement	Aspiration pipette does not contact cells	Limited ability to select cell	Operator skill essential
		Damage to non-biopsied cells?	

48.1.2 Chemical Zona Penetration

In general, mechanical methods for zona penetration are time-consuming and require skillful micromanipulation, possibly making them inaccessible to some IVF laboratories. As an alternative, zona drilling using acidified Tyrode's solution (pH 2.2–2.4) to dissolve the zona glycoproteins has been extensively used and is commercially available from most culture medium manufacturers. This method developed in the mouse embryo model, with the aim of improving fertilization rates with low sperm densities [10], was of limited value when using human oocytes, as an increased fertilization rate was offset against developmental arrest in the zygote, presumably consequent to changes in intracellular pH [11]. With zona drilling, the effect of the acidified Tyrode's solution is localized to a small area of the zona (generally between 20 and 30 µm) using a fine micropipette, with an inner diameter of 5–10 µm. The pipette is placed very close to or in direct contact with the zona pellucida at the desired position and the acidified solution gently expelled from the pipette until the zona thins, and an aperture is drilled (in some cases, the zona can be seen to "pop" as an aperture is made). The flow, facilitated via oil-filled syringe (hydraulic), air-filled syringe (pneumatic), or by using a mouth pipette, must be carefully directed and controlled to limit the size of the zona breach. The human zona is bilayered, and the zona drilling process must be carefully monitored as the outer layer dissolves more rapidly than the inner layer. Moreover, there is great variation in zonae pellucidae both between and within cohorts of human oocytes and embryos. The final diameter of the aperture made will be determined by a combination of the above factors. An excessively large aperture may result in the unwanted loss of blastomeres but, more significantly, may indicate that the blastomeres were exposed to potentially damaging quantities of acid, which could compromise further development. Physiologic pH of the medium was originally maintained by employing phosphate-buffered saline but is now routinely maintained using modified culture medium buffered with either 4-morpholine propanesulfonic acid (MOPS) or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). When the drilling is complete, the micropipette is immediately withdrawn and, if necessary, excess acidified Tyrode's solution aspirated from the biopsy drop.

48.1.3 Noncontact Laser (Thermal Ablation of the Zona)

Since the first PGD cycles reported by the ESHRE PGD Consortium, there has been a marked shift across centers worldwide from zona drilling using predominantly acidified Tyrode's solution [12] to laser ablation of the zona pellucida with the laser now accounting for more than 70% cleavage-stage embryo biopsies [3]. This shift may be more to do with the ease of use and elimination of the need for a double tool holder and batch testing of acidified Tyrode's solutions rather than any measurable improvement in safety or efficacy.

The preferred model of laser is the near infrared (NIR) solid-state compact diode 1.48 μm laser. The advantage of using light as a cutting tool is that it obviates the need for a double tool holder and either disposable or reusable cutting tools. It is extremely precise and, if used appropriately, provides consistent, reproducible, and rapid results. Furthermore, the likelihood of introducing contamination or pH changes in the medium surrounding the embryo is greatly reduced as neither microtools nor reagents are required to dissect the zona. The 1.48 μm diode laser is small but, at the appropriate pulse duration, can emit light at power levels sufficient to cause selective thermal disruption of the zona pellucida glycoproteins and is not absorbed by water. This noncontact laser can be inserted into the body of the microscope on which the manipulations take place or be integrated in a special objective and the beam delivered to the target through the dish.

Since the laser beam travels up through an objective which lies below the sample, localized heating causes denaturation of the zona proteins in a cylindrical spot where the laser beam is focused, and the size of the aperture created is controlled by adjusting the laser pulse duration. The thermal energy created produces a groove in the zona perpendicular to the microscope stage, rather than a circular aperture. However, an "aperture" is produced in the zona at the point at which the zona is perpendicular to the microscope stage (Fig. 48.1). The size of the aperture (or more accurately, the width of the groove at its widest point) created in the zona ranges from 5 to 20 μm and is governed by the pulse irradiation time (ranging from 3 to 100 ms) or the accumulation of pulses along the length of the zona margin. The precision of the laser is illustrated by the fact that drilled mouse and human embryos show no sign of extraneous thermal damage under light or scanning microscopy [13].

Clearly, such equipment may be used for assisted hatching as well as PGD [14], and if used appropriately, there appears to be no detrimental effect of the laser itself on the development to the blastocyst stage or pregnancy rates in animal and human studies [3, 15–17]. However, studies of the immediate effects at the blastomere level in a mouse model [18] and following assisted hatching in a clinical program [19] have shown that the laser can cause damage if used inappropriately. Certainly, if the laser beam is fired in an area in direct contact with a blastomere, its viability is always compromised. However, as the pulse length, and therefore localized heating, is increased, the distance between the laser beam and blastomere required to avoid damage increases [18]. Hence, care is required to drill the zona away from underlying blastomeres and from as far away as possible and also to use minimum pulse lengths to restrict any damaging effects. Several practical guidelines have emerged to ensure safe and effective use of the laser for human embryo biopsy as follows. Wherever possible, a single aperture only should be made for cellular aspiration. Double or multiple apertures may cause problems during embryo hatching as the embryo will attempt to hatch out of multiple openings which could compromise further inner cell mass (ICM) development or lead to increased monozygotic twinning. To generate the desired aperture, several pulses of short duration are preferable to a single pulse of long duration (with higher energy),

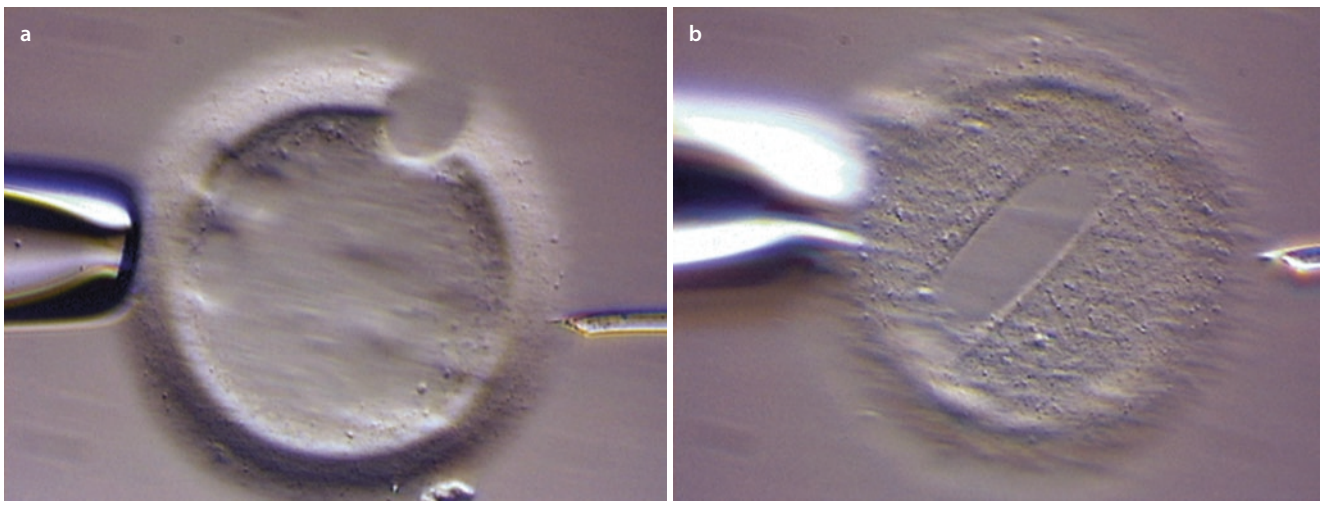

Fig. 48.1 Empty human zona pellucida after thermal ablation using noncontact laser. (**a**) Cross-sectional view as used during biopsy procedure indicating the aperture through which biopsy aspiration pipette is passed. (**b**) The same zona pellucida rotated through 90° to show the path of the laser

which could cause thermal damage. During laser use, it is imperative to maintain the oocyte or embryo as close to the bottom of the biopsy dish as possible to allow a focused beam to ablate the zona pellucida. As the embryo is raised above the dish surface, the beam energy is diffused and can create localized heating or simply prevent effective ablation of the zona. The use of the laser is deceptively simple, and it is imperative that the operator is constantly aware of the possible detrimental effects to the embryo of unnecessary or misplaced ablations.

48.2 Blastomere Removal

Having created an aperture large enough for the safe passage of one or more blastomeres, the operator must select a method for cell removal. The most frequently used method of blastomere removal is aspiration, but other methods have been described and used clinically, although no studies have been conducted to compare their relative safety and efficacy.

48.2.1 Aspiration

If performing aspiration in conjunction with zona drilling using acidified Tyrode's solution, it is easier to use a double tool holder containing a second aspiration micropipette (internal diameter of 30–40 μm depending on the cell size) filled with biopsy medium [2] rather than changing the micropipette in a single tool holder for each biopsy procedure. A single micropipette may be used for both drilling and subsequent aspiration, but care is needed to prevent overexposure to acid [20, 21]. Any advantage accrued in terms of speed of the procedure may be offset by potential damage as a result of overexposure to acid.

A typical procedure for cleavage-stage biopsy using laser and blastomere aspiration is illustrated in Fig. 48.2. Briefly, following laser ablation of the zona pellucida adjacent to the blastomere selected for analysis, the blastomere is aspirated by gentle suction using a finely polished "sampling" pipette. The aperture may be sited adjacent to either a selected blastomere or a sub-zonal space between blastomeres. The pipette is placed through the aperture, close to the blastomere to be aspirated. By gentle suction, the blastomere is drawn into the pipette while the pipette is withdrawn from the aperture. The aperture of the sampling pipette is critical for successful biopsy. If the internal diameter is too large for the cell being removed, the pipette will have little purchase on that cell and may result in unwanted suction on non-biopsied cells. Conversely, an undersized pipette will cause the biopsied cell to be squeezed unnecessarily, resulting in blebbing on the cell membrane and ultimately lysis, which will likely reduce the chances of a successful diagnosis in that embryo. Similarly, use of a holding pipette with an internal diameter of 30 μm (i.e., larger than a regular ICSI holding pipette) ensures safe and reliable suction on the zona particularly during difficult biopsies.

Once the blastomere is free of the embryo, it is gently expelled from the sampling pipette. Following biopsy, the embryo should be rinsed in culture medium at least twice to remove residual embryo biopsy medium before returning to the culture. The blastomere should be washed extensively in handling medium before proceeding to the analysis.

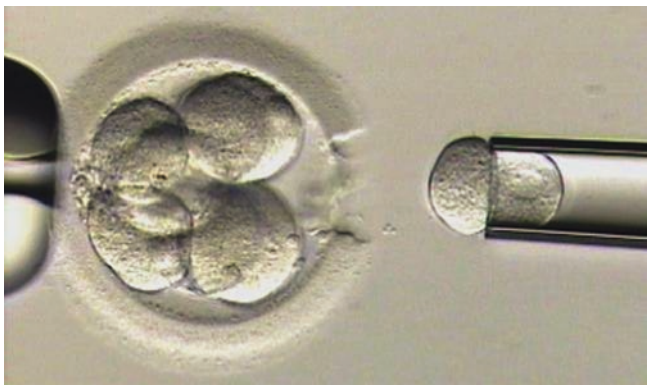

Fig. 48.2 Human cleavage-stage embryo from which a single blastomere with a single visible interphase nucleus is being removed by micromanipulation after laser ablation of the zona pellucida

48.2.2 Alternative Methods of Blastomere Removal

In the extrusion method, after zona pellucida drilling, the blastomere is extruded through the aperture by pushing against the zona at another site (usually at 90° to the aperture) using a blunt pipette [6]. The slit in the zona pellucida can be introduced using mechanical means, chemical (acidified Tyrode's solution) exposure, or laser ablation as described above.

Another variation in the method of cell removal involves fluid displacement whereby culture medium surrounding the embryo is used to displace individual cells following a zona breach. This method was pioneered in mouse embryos by introducing a slit in the zona with a sharpened needle and, through a second puncture site, injecting medium to dislodge the blastomere through the first puncture site [7]. This method requires the production of two separate apertures and considerable skill to displace the blastomere of choice but has been successfully modified for clinical application [22]. A challenge common to both of these methods is to ensure that only the selected cell or cells are removed.

48.3 Practical Considerations for Embryo Biopsy

48.3.1 Preparation Prior to Biopsy

ICSI is still recommended for all PGD cases involving DNA amplification to reduce the chance of paternal contamination from extraneous sperm attached to the zona pellucida or non-decondensed sperm within blastomeres [23]. Similarly,

as far as is practically and safely possible, all cumulus cells should be removed before biopsy as these cells can contaminate both fluorescence in situ hybridization (FISH) and polymerase chain reaction (PCR)-based diagnoses. Embryo and blastomere identity (individual drops or dishes) should be checked throughout the procedure so that diagnostic results can be reliably linked to specific embryos [23–26]. The use of standard IVF culture medium during biopsy is acceptable, but its effectiveness may be highly dependent upon the developmental stage of the embryo biopsied with compacting eight-cell embryos proving more difficult to biopsy. Commercially produced calcium- and magnesium-free (Ca^{2+}/Mg^{2+}-free) medium which temporarily reverses calcium-dependent cell–cell adhesion [27] is widely available and is used by many centers for routine clinical biopsy with the benefit of reducing the frequency of cell lysis [23] combined with a shorter time needed to perform the biopsy procedure.

48.3.2 Timing of Biopsy

Most of the cleavage-stage biopsy takes place on the third morning following insemination, although the exact timing varies according to timings of procedures in different laboratories and may be patient-specific or even cohort-specific for particular patients. One variation, allowing more time for genetic analysis, is to alter the timing of ICSI to allow cleavage-stage biopsy at the same embryonic stage but late on day 2 since biopsy at earlier cleavage stages on day 2 may adversely affect embryo development [28]. In cases where retarded development is observed, the possibility of delaying the biopsy procedure to allow diagnosis of a larger proportion of the embryo cohort should be considered. The use of Ca^{2+}/Mg^{2+}-free medium has also facilitated later biopsy (i.e., beyond eight-cell stage) making the laboratory timings more flexible. Furthermore, the increased use of sequential media and blastocyst culture and transfer has led to the routine delay of transfer until day 4 or more commonly day 5. This extended culture period allows additional time for diagnostic analysis and allows more opportunity to preferentially select the most developed embryos for transfer with the aim of improving pregnancy and implantation rates during a fresh transfer cycle and facilitating elective single embryo transfer to reduce multiple pregnancy rates [29].

Most laboratories exclude very poor-quality embryos or those not reaching a predefined cell stage from the embryo biopsy procedure. Of the centers surveyed, most will consider only embryos at the five-cell stage and beyond only for biopsy [12]. Biopsy at the four-cell stage in mouse results in a distorted allocation of cells to ICM and trophectoderm and abnormal postimplantation development [30], while human embryos biopsied on day 2 show cleavage rate retardation and smaller blastocysts [28]. Conversely, four-cell stage human embryos surviving freeze–thaw procedures with the loss of one or more blastomeres can develop, implant, and result in live birth, albeit at a reduced rate compared with nonfrozen embryos [31, 32]. Stringent biopsy policies have the benefits that fewer embryos need to be biopsied and fewer cells prepared and tested with only developmentally competent embryos considered with relatively little loss in pregnancy potential for that cycle. On the down side, an opportunity to identify genotypes on a full cohort of embryos may be lost.

48.3.3 Number of Cells to Remove During Cleavage-Stage Biopsy

In deciding how many cells to biopsy from cleavage-stage embryos, it is axiomatic to balance diagnostic accuracy with the potential to implant and develop, which is progressively compromised as a greater proportion of the embryo is removed [33]. There is no consensus on the number of blastomeres that can be safely removed during cleavage-stage embryo biopsy. In many centers, a second blastomere is removed from embryos having seven or more cells regardless of the type of analysis involved, but this approach has been criticized as compromising the implantation potential of the biopsied embryo based on extrapolation from frozen–thaw embryo implantation rates [32]. The decision to remove one or two cells is based on many factors including the embryo cell number and the accuracy and reliability of the diagnostic test used. Removal of two cells should only be considered on embryos with six or more cells [34]. While the removal of two blastomeres decreases the likelihood of blastocyst formation, compared with the removal of one blastomere, day 3 in vitro developmental stage is a stronger predictor for day 5 developmental potential than the removal of one or two cells. The biopsy of only one cell significantly lowers the efficiency of a PCR-based diagnosis, whereas the efficiency of the FISH PGD procedure remains similar whether one or two cells are removed [35]. However, a recent trial demonstrated that live birth rate was compromised at a level of 1 birth for every 33 cycles of two-cell embryo biopsy suggesting that, ideally, one-cell biopsy should always be performed unless the diagnostic test is suboptimal [36].

In the case of lost or anucleate blastomeres and failed diagnosis, rebiopsy of embryos is possible, but embryo cell number and timing of rebiopsy should be considered to avoid excessive harm to the embryo. Although technically challenging, the original zona breach site should be accessed to prevent later problems, including monozygotic twinning, possibly the result of embryos hatching via multiple sites. No specific recommendations for time limits for embryos out of the incubator are available, but ideally, biopsy should be performed as quickly as possible (certainly less than 5 min in total and, ideally, 1–2 min per embryo) to ensure pH, temperature, and osmolality are maintained. A documented record for biopsy timings should be maintained for quality assurance purposes [26, 27].

48.3.4 Success Rates After Biopsy

The reliability of cleavage-stage biopsy has now been established in many centers, and in a recent ESHRE PGD Consortium report, the efficiency of successful embryo biopsy is 98% in over 150,000 cleavage-stage embryos in clinical PGD cycles [3]. Pregnancy rates after PGD are notoriously difficult to assess between different indications and centers. Nevertheless, in the largest series analyzed in detail to date, mostly following cleavage-stage biopsy, pregnancy rates are only 22% per oocyte retrieval and 30% per embryo transfer on average [3]. The reasons for the apparently low success rates are manyfold but unsurprising considering that a proportion of embryos cannot be transferred because they are diagnosed as affected, and in many countries, the number of embryos transferred is limited to a maximum of two. To demonstrate the possible detrimental effects of embryo biopsy alone, one would need to conduct a clinical trial involving biopsied and non-biopsied embryos which would be transferred after selection on purely morphological grounds post-biopsy (i.e., without any genetic selection). Such a trial could be considered unethical. However, data from a recent trial provides some insight into the possible detrimental effects of biopsy with a reduction in implantation potential evident in undiagnosed biopsied embryos compared with non-biopsied control embryos [37, 38].

It is well established in mammalian embryos that as an increasing proportion of the embryo is removed or destroyed before transfer, implantation and fetal development rates decline, suggesting a lower limit of embryo mass compatible with implantation and development [39]. Reduction of 50% or more of the cell mass frequently results in cell proliferation in the absence of normal differentiation; thus, it is important to minimize the cellular mass removed at biopsy. However, cell reduction within this limit is compatible with normal embryo metabolism, blastocyst development, and fetal growth, while cell numbers in the trophectoderm (TE) and ICM of blastocysts were in proportion to the cellular mass removed at biopsy, making cleavage-stage biopsy for PGD a viable option [40]. Hence, human cleavage-stage biopsy is delayed until just before the beginning of compaction, the process of intercellular adhesion, and junction formation, which progressively makes the removal of blastomeres more difficult and eventually impossible without causing damage to the embryo. Generally, cells identified as having completed the third cleavage division (on the basis of their size) are selected for biopsy. Theoretically, therefore, each blastomere removes only one-eighth of the cellular mass of the embryo. As zona drilling for assisted hatching may be beneficial for some indications [41], it is also possible that the hatching process itself offsets to some extent the adverse effects of reducing the cell mass of the embryo.

In frozen embryo transfer (FET) cases, viable pregnancies are routinely achieved, albeit at a reduced rate compared with fresh transfer cycles. Moreover, no increase in fetal abnormalities has been reported following the transfer of cryopreserved embryos in which some cells have been destroyed by freezing and subsequent thawing of cleavage-stage embryos [42, 43]. Indeed, estimates of the loss of implantation potential have been made based on outcomes following FET involving cleavage-stage embryos with one or more nonviable cells after thawing [31, 32], although it is clear that the growth rate of viable cells may be more important than the loss of cells per se [31, 42]. It is now apparent that cleavage-stage biopsy should be considered a "cost" to the embryo, and this must always be weighed against the potential benefit to the embryo of any diagnostic testing.

48.3.5 Selection of Cells in the Cleavage-Stage Embryo

Biopsy at cleavage stages is based on the principle that at these stages, the blastomeres remain totipotent and equivalent such that the removal of a single blastomere will (a) provide a representative sample of the entire embryo and (b) compromise the embryo only to the extent of one-eighth of the embryo mass rather than removal of a developmentally crucial blastomere. The importance of selecting a blastomere with a single visible interphase nucleus cannot be stressed enough (Fig. 48.2). Aside from the increased diagnostic efficiency observed in mononucleated blastomeres [44], mononucleation is a marker for and directly correlates with implantation potential [45]. Nevertheless, embryos containing blastomeres, all of which have no visible nucleus, should still be considered for biopsy as nuclear material is likely to be present and should yield results in molecular tests [44]. After micromanipulation skills, blastomere selection is probably the most challenging aspect of effective cleavage-stage biopsy. Time spent in careful examination of the embryo and orientation to selectively remove specific blastomeres is essential to attain the high diagnostic efficiencies required for clinical effectiveness. The reasons for this are that, first, an interphase nucleus is essential for FISH analysis since the nucleus is prepared on a slide by a process of cell lysis in which individual chromosomes from a metaphase plate may not be visible and are likely to be lost during cell preparation [46]. Second, post-zygotic chromosomal mosaicism arising during cleavage is known to be associated with nuclear abnormalities [47]. The exception is binucleate blastomeres, in which there are two normal-sized nuclei. In most cases, these are generated through failure of cytokinesis, and both nuclei contain the normal diploid chromosomal complement for that embryo [48]. In general, multinucleate cells should not be selected at biopsy if FISH analysis for aneuploidy detection follows, and the removal of mononucleate cells only is recommended [23]. The dilemma with this selection procedure is that in a chromosomally mosaic embryo (which contains significant proportions of both normal diploid and aneuploid cells), removal of only mononucleate cells (which are more likely to be chromosomally normal) may result in only the chromosomally abnormal multinucleated cells

remaining in the embryo. For accuracy during FISH-based diagnosis, it is advisable to only use bi- or multinucleated cells as a last resort in the absence of mononucleated cells. This may be less critical for PCR-based testing in which presence or absence of a specific parental chromosome is important rather than copy number per se. However, even with careful blastomere selection, diagnostic efficiency is not 100%, and aneuploid results are common even in mononucleate blastomeres primarily as a result of chromosomal loss and mitotic nondisjunction, leading to chromosomal mosaicism [48]. Biopsy of two nucleated blastomeres is only possible in good-quality embryos at a sufficiently advanced stage, such that even with a two-cell biopsy policy, a mixture of embryos with one or two blastomeres for analysis is common [34]. Where possible, one of the smaller blastomeres should be selected to minimize the reduction in mass, and the relative sizes of cells may provide an indication of recent mitosis. This may also reduce the risk that a cell in metaphase will be taken; the chromosomes of which could be lost during the fixation process.

48.3.6 Safety of Cleavage-Stage Embryo Biopsy

As with any micromanipulation procedure involving human gametes or embryos, every reasonable precaution should be taken to minimize cellular damage and stress during the procedure. General precautions include the correct installation, calibration, and maintenance of all micromanipulation equipment (particularly the laser). In advance of all clinical procedures, one should ensure that all appropriate reagents and micromanipulation tools are available, sterile, and within their expiration date. Biopsy and cell preparation should be performed by a suitably qualified and trained person. Regular reviews of key performance indicators [26, 27] such as the rate of biopsied cell lysis, post-biopsy survival, morphology, and cell numbers of untransferred embryos provide an indication of the possible harm as a result of biopsy as do pregnancy rates after biopsy—particularly those not progressing beyond the biochemical stage. Clearly, effects on postimplantation development should also be closely monitored as any increase in fetal malformations or congenital abnormalities would be unacceptable. To date, studies of pregnancies and children born after PGD have identified no significant increase in abnormalities above the rate seen in routine IVF [3, 49–51]. The main problem in terms of diagnostic efficiency with cleavage-stage biopsy is the presence of chromosomal mosaicism, which is reported to occur in up to 80% cleavage-stage embryos [52–54]. A full discussion of the impact of chromosomal mosaicism on the accuracy of PGD is beyond the scope of this review, but its impact on both diagnostic accuracy and clinical effectiveness of PGD can be significant. Mosaicism is thought to be the primary reason for the high rate of false positives depleting the pool of chromosomally "normal" embryos for transfer and hence significantly lowering the chance of live birth following preimplantation genetic diagnosis of chromosomal aneuploidy (PGS) compared with controls in a recent randomized controlled trial [37]. However, the impact of mosaicism on the misdiagnosis rate when performing PGD by PCR analysis appears to be less significant [55]. While polar body biopsy appears to offer a solution to the problem of mosaicism by focusing on maternal mutations and/or meiotic errors—acknowledged to be the main source of aneuploidy in human IVF—the approach does not address either paternal mutations, meiotic errors, or post-zygotic errors arising in the embryo [2]. Blastocyst biopsy has been proposed as a solution to the problem of cleavage-stage mosaicism and has been used successfully in the clinical setting [56–59]. While its use is becoming more widespread, it is still unclear whether or not any residual mosaicism reduces diagnostic accuracy and hence clinical effectiveness at this developmental stage, although its use in aneuploidy detection appears to be superior to that of cleavage-stage biopsy and analysis [59]. Since blastocyst biopsy focuses on only the most developmentally competent embryos within a cohort, diagnostic costs may be lower, and the outcomes per biopsied embryo improved; however, a comprehensive diagnosis of the embryo cohort is not possible.

As an alternative to blastocyst biopsy, it is possible to coculture blastomeres biopsied at cleavage-stages with the biopsied embryo [60]. Over a period of 3 days, division and development of the biopsied blastomere mirrors the behavior of the parent embryo. Hence, if the embryo reached the blastocyst stage, in most cases, the blastomere divided and developed into a small TE vesicle. On average, those blastomeres that divided and formed these vesicles divided two or three times, resulting in an average of 5.6 ± 0.6 (n = 13) cells for single eight-cell-stage blastomeres and 9.1 ± 1.1 (n = 11) cells where two blastomeres were biopsied and encouraged to form a single morula. In this approach, the behavior of the cleavage-stage biopsy in vitro could predict the potential for the biopsied embryo [61], thereby avoiding the difficulties and damage of biopsy at the blastocyst stage itself and the possibility of having no embryos to biopsy, if one elected for blastocyst biopsy alone.

48.3.7 Cryopreservation of Embryos Following Cleavage-Stage Biopsy

A major challenge at present is to develop an effective standardized method for cryopreservation of biopsied embryos. Attempts to use established protocols either in the mouse model or in humans have shown extensive damage after thawing, presumably because of the loss of protection from ice crystals in the medium provided by an intact zona pellucida [62, 63]. However, recently, several improved slow-freezing protocols for biopsied cleavage-stage embryos have been reported in which damage is much reduced [64, 65]. However, following successful application in animal models, vitrification looks set to replace slow freezing for both cleavage- and blastocyst-stage embryos after polar body or embryo

biopsy [59, 66–68]. With the high rate of multiple pregnancies reported after PGD, it is imperative to develop effective methods of cryopreservation that will (1) allow storage of unaffected embryos for later transfer so that the numbers transferred can be limited to two or even single embryo transfers and (2) provide additional time to perform more extensive diagnostic tests.

48.4 Future Developments

With the introduction of quality management systems and accreditation in IVF laboratories [25–27], safer and more effective biopsy should be achieved through agreed definitions of successful and safe biopsy, standardized training and procedures, and validation of new techniques as well as calibration of new and existing instruments such as the laser. It has become clear that embryo biopsy, as with any form of invasive testing or manipulation, exacts a cost to the embryo in the form of cellular depletion and metabolic stress. Thus, it is imperative to assess the potential benefit to the embryo itself in terms of improved selection or disease-free status before performing embryo biopsy. However, in the future, it may be possible to diagnose inherited diseases or chromosomal imbalance in early human embryos by noninvasive analysis of the secretome or metabolome in spent culture medium, an advance which would shift the cost–benefit ratio heavily toward potential benefit. At present, noninvasive analyses are likely to be used as an adjunct to assess embryo quality and viability with the genetic test requiring biopsied cellular material [69]. For the time being, it is critical that the diagnostic laboratory optimizes the use of each single biopsied blastomere. Whole genome amplification is one such optimization allowing the testing of multiple loci, repeat testing, sample sharing for external quality assessment, and archiving for later assessment of additional loci. A sufficiently large amount of DNA is generated following this process such that microarray-based testing is possible from a single cell for detection of either chromosome, single gene, or a combination of both [70].

In conclusion, blastomere biopsy of human cleavage-stage embryos remains the most commonly performed form of biopsy for genetic diagnosis of the early embryo. The various techniques used appear to be largely safe in terms of pregnancy outcomes and health of children, but it is clear that biopsy of even a single blastomere has some degree of cost to that embryo, manifested as reduced implantation potential. For this reason, the benefit of the possible diagnosis should always be weighed against the "cost" of the embryo biopsy. The noncontact laser has largely overtaken other methods for breaching the zona pellucida and has made embryo biopsy techniques accessible to any embryology laboratory with micromanipulation capabilities. The comparative ease with which the laser may be used should be carefully considered to avoid any inadvertent damage to embryos through misuse. The combined introduction of the laser and large diagnostic laboratories providing PGD services on a satellite basis to embryology laboratories worldwide has greatly improved access for patients to PGD services. The biggest change in practice in future is a shift away from cleavage-stage biopsy, in which chromosomal mosaicism is an occupational and biological "hazard," toward more trophectoderm biopsy from blastocysts in which greater accuracy and diagnostic reliability is predicted.

Review Questions

1. Please define what are the objectives of embryo biopsy.
2. Please describe the required settings for cleavage-stage embryo biopsy.
3. Please explain what the options are to get the zona pellucida open and have access to embryonic cells/blastomeres.
4. Please describe the procedure of embryo biopsy, the removal of the blastomere(s), and its preparation for genetic testing.

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Current andNovel Methods forChromosome Testing

Sarthak Sawarkar and Santiago Munné

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More than 50% cleavage and blastocyst stage embryos produced in vitro are found to be chromosomally abnormal, increasing up to 80% in women over 42 years of age [1]. While some abnormal embryos arrest during culture, most do not. Embryos with numerical chromosomal abnormalities are usually not compatible with either implantation or birth, with and up to 70% of spontaneous abortions being chromosomally abnormal, clearly highlighting the detrimental effects of aneuploidy. Thus, we hypothesized that the selection of normal (euploid) embryos for transfer should improve the success rate of IVF [2]. This process of selection against aneuploidy is known as preimplantation genetic diagnosis (PGD) of aneuploidy (PGD-A) or preimplantation genetic screening (PGS).

49.1 PGS Version 1 andIts Limitations

Early strategies for PGS involved using fluorescent in situ hybridization (FISH) to analyze day-3 blastomere biopsy or polar bodies biopsied from oocytes and/or zygotes [3]. FISH allowed the analysis of anywhere between 5 and 12 chromosomes per sample but a complete evaluation of the chromosome complement. Even with this limited coverage, FISH detected more than 80% of chromosomally abnormal embryos. Another limitation of FISH was the need of fixing the cells, which required dexterity, and some methods were better than others [4] but not used widely. More importantly, cleavage-stage biopsy required as much skills, and although experienced centers showed improved results in clinical randomized trials (CRTs) [5], on the other hand, it could reduce implantation potential by half [6–8]. Thus, while some studies showed an improvement in implantation rates, reduction in spontaneous abortions, and take-home-baby rates [9–18], others showed no improvement or even a detrimental effect of PGS [6, 19–21].

The most likely cause explaining the inter-center differences in PGD outcomes are variations in biopsy and genetic techniques employed. For example, one of the studies showing detrimental effects of PGD used a two-cell biopsy [22]. However, the same group later reported a detrimental effect of a two-celled biopsy compared to the one-celled biopsy from cleavage-stage embryos [23]. Biopsying even a single cell in sub-optimal laboratory conditions has been shown to be extremely damaging to the embryo potential. Mastenbroek et al. (2007) reported a very high rate of diagnostic failure (20%) resulting in many of the embryos included in the study being transferred without a diagnosis. The implantation rate of these non-diagnosed embryos was 59% lower than the control. In this case, the only difference between the control and the test group appears to be the biopsy. This suggests that embryo viability was drastically reduced by sub-optimal biopsy procedures used in that clinic.

The second factor in obtaining good outcomes with FISH is a low error rate. Error rates varied widely between PGD laboratories, 2–7% [10, 24, 25] to 40–50% [26, 27], due to varying practices in PGD laboratories in the steps after the biopsy of the embryo. Higher error rates have been shown to decrease implantation rates [28]. When FISH was performed using appropriate, well-validated methods, it detected over 80% of chromosome abnormalities detected by a CGH [9, 29].

The primary argument made to explain the detrimental effect of PGS by the groups with poor results was mosaicism. However, although mosaicism is common in cleavage-stage embryos, most of these embryos have chromosome abnormalities in every cell [10]. In this case, the biopsied cell is chromosomally different to the remaining cells in the embryo but still correctly classified as abnormal. However, bad fixation methods could increase the error rate dramatically. With good fixation methods, mosaicism produced about 7–10% misdiagnoses [10].

Overall, although some PGD laboratories had consistent results with FISH and cleavage-stage embryos and improved outcomes, the field of PGD/PGS has moved away from those techniques. Most PGT is now being performed at blastocyst stage on days 5–6 of development, which seems to be more resilient to technical manipulation [8]. In addition, FISH has been replaced by more comprehensive methods of DNA analysis, which detect all numerical chromosomal abnormalities (except polyploidy). These techniques constitute what we call PGS version 2.

49.2 Biopsy Techniques

For PGD, oocytes obtained during ART are stripped from their surrounding cumulus cells, because these cells can potentially provide a source of non-embryonic DNA, which could cause contamination during PGD [30, 31]. Intracytoplasmic sperm injection (ICSI) is used most routinely to fertilize oocytes in order to reduce the rate of fertilization failures and also to prevent any contamination caused by DNA from residual sperm attached to the zona pellucida [31, 32]. Post fertilization, the embryos can be biopsied at different stages of their development, and one or more cells can be retrieved or polar body biopsy can be performed to obtain the first and second polar bodies extruded from the oocyte [22].

49.3 Polar Body Biopsy

Genetic material for PGD can be obtained pre-fertilization by the removal of the first polar body from the mature (metaphase II) oocyte. The first polar body can be used to deduce the genotype of the oocyte before fertilization, while upon fertilization, the second polar body can also be obtained and tested [33]. Polar body PGD was first applied by Verlinsky and colleagues [34] for PGD and Munné et al. for PGS [2], and the accuracy of the method has been shown in a large number of PGD cases [35]. It should be noted that PGD for single gene disorders requires that both the first and second polar body are tested, since meiotic recombination can lead to unclear (heterozygous) results in the first polar body. Polar

body biopsy was also widely used for aneuploidy screening of the oocytes [36]. The benefit of this method is that it does not involve biopsy of the embryo and is apparently non-invasive (although the egg can be harmed during second PB if a bridge between the metaphase and PB persists). Furthermore, in some countries (e.g. Germany), where testing of embryos is forbidden or highly restricted, it is still the main choice for obtaining material for PGD. However, polar body biopsy only provides information regarding the oocyte genotype [37], and any chromosomal aneuploidies inherited from the sperm or arising post-mitotically will not be detected [38].

49.4 Cleavage-Stage Biopsy

Cleavage-stage biopsy used to be the most popular method used in PGS but now has been replaced mostly by blastocyst biopsy.

Cleavage-stage embryo biopsy is performed by breaching the zona pellucida with either acid Tyrode's solution or laser or mechanically [39]. At the day-3 stage, the embryo typically contains 6–12 cells which are totipotent [33]. One or two blastomeres are extracted and used for PGD. The removal of two cells has proven to be clearly detrimental decreasing the implantation potential and reducing the rate of blastocyst formation [22, 40]. Suitable embryos are selected and transferred to the uterus of the woman on day 4 or day 5 post fertilization. Compared to polar body biopsy, day-3 biopsy permits checking the embryo for genetic disorders inherited from both parents and mitotic chromosomal imbalances. A challenge for cleavage-stage and blastocyst biopsy is mosaicism, or the presence of two or more distinct cell lines with different chromosomal constitutions in the same embryo that occur due to errors that happen during mitosis after zygote formation – especially during the first three mitotic divisions [41–44]. The incidence of mosaicism in cleavage-stage embryos, assessed through FISH, was about 30% [10] and with use of CCS methods was similar or higher [45, 46]. Therefore, a single blastomere biopsied from a day-3 embryo might not always be representative of the rest of the embryo. However, studies on mosaic embryos have shown that a majority of day-3 mosaic embryos have all their cells abnormal and the error rate caused by them is about 7–10% [10]. Mosaicism in spontaneous miscarriage specimens is seen at a degree of <10% [47], while for first trimester, the incidence in ongoing pregnancies is even lower (1–2%) [48]. It therefore seems that mosaicism disappears prior to the period of first trimester through mechanisms that involve loss of mosaic embryos or euploid cells taking over abnormal cells [49].

49.5 Blastocyst Biopsy

Blastocyst culture, vitrification, and blastocyst biopsy techniques have improved considerably in the last 10 years and are an essential aspect of ART [50]. Frozen cycle transfers accounted for almost 30% of transfers in North America, while over 50% cycles in Nordic countries are frozen transfers and is routinely performed for cases undergoing PGS.

The most popular option nowadays for obtaining genetic material from the embryo is through blastocyst biopsy reached at days 5–6 of development. At this stage, the inner cell mass that gives rise to the fetus has differentiated from the trophectoderm, which later develops into extraembryonic tissues [33]. Approximately 3–10 cells are removed during biopsy at the blastocyst stage. Biopsy of the trophectoderm is advantageous because no cells are extracted from the inner cell mass, and in contrast to the other two biopsy strategies, this method obtains multiple cells for carrying out PGD/PGS, which leads to improved accuracy. Moreover, blastocysts are robust compared to the earlier embryonic stages and tolerate biopsy better than cleavage-stage embryos [8]. Two drawbacks of this method are that only about 50% of the embryos growing in vitro reach the blastocyst stage [51] and the time left for diagnosis after biopsy is limited since the embryos need to be transferred by day 6 [30]. However, the introduction of vitrification as a method to cryopreserve blastocysts [52] and the high survival rates obtained even after biopsy [53] make blastocyst biopsy increasingly attractive for PGS. Theoretically, cryopreservation of biopsied blastocysts allows for an unlimited amount of time for PGS to take place. Furthermore, vitrification of blastocysts and their transfer later on during a natural ovulatory cycle rather during a hormonally stimulated cycle has been found to result in a better clinical outcome [54]. As in cleavage-stage embryos, mosaicism is detectable in blastocysts [41, 47]; the incidence however is found to be considerably lower (4–35%) depending on the technique used [41]. Through data collected from different studies, it is suggested that a proportion of mosaic embryos undergo developmental arrest before reaching the blastocyst stage [47, 55]. This explains the lower rates of mosaicism at the blastocyst stage when compared to day-3 embryos, while it also suggests that culture to the blastocyst stage might be advantageous since it would identify embryos of increased developmental competence. Furthermore, the possibility of misdiagnosis is believed to be considerably lower since, now with NGS, they can be identified when the number of abnormal cells is between 20% and 80% [56–58], preventing a large degree of the transfer of mosaic embryos, which would possibly have a negative effect on IVF outcome.

Blastocyst biopsy is not completely benign, and some forms may be detrimental. For instance, there are two studies reporting adverse effect on implantation potential if 10 or more cells are biopsied [59]. Also there are two modalities of blastocyst biopsy, one described by [60] in which the zona is breached on day 3 and the herniating TE is biopsied on days 5–6 and the other described by [61] in which the blastocyst zona is breached on days 5–6 and then the TE is biopsied. There are no studies yet comparing which method is better; plus within each method, the number of laser pulses vary from lab to lab.

49.6 Screening Techniques Used inPGS v2

A variety of PGS v2 technologies are currently being used in PGS laboratories for comprehensive chromosome screening. In this part of the chapter, we will cover CGH, array CGH, SNP arrays, and next-generation sequencing (NGS) techniques that are routinely being used for testing embryos. The advantages and disadvantages for every technology will be discussed alongside the technique itself. Microarray CGH (array CGH or aCGH) and single-nucleotide polymorphism (SNP) microarrays can be used for comprehensive chromosome analysis of single cells from day-3 biopsy and day-5 embryos. NGS, however, has been validated only for blastocyst biopsies.

49.6.1 CGH

The first technique employed was comparative genome hybridization (CGH), a molecular cytogenetic technique that allowed for the performance of a global assay to find chromosomal gains and losses in embryos [62]. CGH was initially used in day-3 embryo biopsies. However, CGH as a technique has several pitfalls. CGH is labor intensive and time consuming. This makes day-3 biopsy and day-5 transfers (fresh transfer) logistically infeasible. During the early days of CGH, embryo freezing was not very efficient and the survival rates of embryos post thawing were low. Hence, CGH was temporarily abandoned and not reused till vitrification was a standard procedure. CGH has been clinically applied to blastocyst biopsies and polar body biopsies. Vitrification along with CGH significantly improved the implantation rates from 46.5% to 72.2% in cycles with screening, with almost 100% of blastocysts surviving the embryo biopsy [53].

49.6.2 aCGH

Array CGH (aCGH) is widely used for the cytogenetic analysis of prenatal and postnatal samples [63–66] since it is rapid and cost-effective and allows chromosomal regions to be screened at high resolution. Several types of aCGH platforms are available for the purposes of aneuploidy screening. The variety most commonly used for the purpose of PGD utilizes bacterial artificial chromosome (BAC) probes, about 150,000 bp in length, covering all chromosome bands and giving a 4 MB or lower resolution. Even higher resolutions are achievable but not recommended since, at that level, the difference between clinically significant duplications/deletions and normally occurring copy number variations is less clear. The microarray validated for PGD had 4000 probes and thus covered ~25% of the genome sequence [67]. aCGH provides a quantitative analysis based on comparing the relative amount of DNA from two different sources, one from the clinical sample (e.g., a cell from an embryo) and another from a chromosomally normal individual. DNA samples from the two sources are differentially labeled and hybridized to either metaphase chromosomes (CGH) or probes on a microarray (aCGH). In the case of aCGH, each probe reveals the relative amounts of these two DNAs at a single chromosomal site. Since multiple copies of each probe are placed on the microarray and each chromosome is tested at several distinct loci, the diagnosis is very accurate.

Chromosome imbalances (aneuploidies, unbalanced translocations, deletions, and duplications) are easily detected by aCGH, but a limitation of these approaches is that diploidy cannot be distinguished from changes involving loss or gain of an entire set of chromosomes (e.g., haploidy, triploidy, tetraploidy, etc.). How important is this? We report (personal communication) that about 7.7% (n = 91,073) of the supposedly 2PN embryos tested by FISH were polyploid or haploid, but the majority of them had additional abnormalities detectable by array CGH, and only 1.8% of all embryos were homogeneously polyploid or haploid. Furthermore, of those embryos, the majority arrested by day 4, leaving only 0.2% of developing embryos uniformly polyploid or haploid. This suggests that failure to detect polyploid embryos may rarely lead to a misdiagnosis but is unlikely to have a significant impact on the clinical efficacy of the screening using aCGH.

aCGH has been used in multiple PGS studies [53] including a randomized clinical trial showing a significant improvement in pregnancy rates over no PGD [68].

49.6.3 SNP Arrays

Single nucleotide polymorphisms are areas of the genome where a single nucleotide in the DNA sequence varies within the population. Most SNPs are biallelic, existing in one of two forms, and are found scattered throughout the genome. By determining the genotype of multiple SNPs along the length of each chromosome, a haplotype (a contiguous series of polymorphisms on the same chromosome) can be assembled. This ultimately allows the inheritance of individual chromosomes or pieces of chromosomes to be tracked from parents to embryos. Current SNP microarrays simultaneously assay hundreds of thousands of SNPs, while utilizing powerful software to distinguish how many copies of each chromosome was inherited by an embryo [67, 69, 70].

Chromosome screening methods (CGH, aCGH, SNP microarrays, and NGS) rely on whole genome amplification (WGA) to amplify DNA from the single cell or small number of cells removed from a developing embryo [71]. CGH can be performed in combination with a variety of WGA methods; however, SNP microarrays are more sensitive to the type of amplification technique used and are not compatible with all methods. WGA methods like multiple displacement amplification (MDA), GenomePlex, and PicoPlex are most commonly used for SNP microarrays. These amplification methods allow for better overall coverage of the genome compared with earlier WGA methods (e.g., degenerate oligonucleotide primed PCR) and are less inclined to preferentially amplify some parts of the genome while leaving others unamplified or under amplified. While the technologies differ greatly, both types of arrays (CGH-based and SNP-based) and NGS are trying to

answer the same underlying question: how many copies of each chromosome is present in a sample? The small size of the SNP array probes can lead to poor hybridization efficiencies and low signal intensities for individual probes. This factor, coupled with the failure of WGA methods to amplify the entirety of the genome, can lead to many probes yielding no result (i.e., a low "call rate"). Also, allele dropout (ADO) and/or preferential amplification (PA) of one SNP allele versus another can lead to a great deal of "noise" in the system, which requires sophisticated interpretation. Several methods for the cleanup of data from SNP microarrays have been developed: (1) qualitative methods, looking only at the inheritance of specific SNPs and requiring comparison with parental DNA samples; (2) quantitative approaches, assessing only the intensity of SNP calls; and (3) techniques combining qualitative and quantitative methods, using both SNP intensity calls and inheritance patterns. For qualitative approaches, it is necessary to assess parental DNA prior to clinical embryo testing. The key requirement is the deduction of the four parental haplotypes for each chromosome. Embryo testing is then focused on detecting the individual parental haplotypes, revealing how many chromosomes were inherited from each parent, i.e., karyomapping [67]. This approach has the disadvantage that mitotic abnormalities, in which only two haplotypes are present in a trisomy (i.e., caused by duplication of one of the two chromosomes in the embryo after fertilization), will not be detected [72]. This can misdiagnose a substantial amount of embryos since 30% of aneuploid embryos contain mitotic abnormalities (mosaics). Qualitative SNP approaches are still used for PGD of gene defects with the karyomapping approach [43] but not for PGS alone (Table 49.1).

A quantitative approach compares the intensity of each SNP against the other SNPs. A purely quantitative approach for aneuploidy screening may not require parental testing ahead of the cycle; however, this approach would not be compatible with combination testing of single gene defects with aneuploidy screening (discussed below). This approach is currently the least developed. A qualitative/quantitative approach has also been applied clinically and probably can obviate the issues mentioned above for purely qualitative or quantitative approaches [69, 70]. All these approaches still share one limitation and that is the diagnosis of tetraploidies. In a tetraploid cell, only two haplotypes are present (i.e., a postmeiotic duplication of a euploid cell); therefore, all SNPs will have the same intensity. SNP-based microarrays do offer some advantages over aCGH: (a) if qualitative analysis is employed, SNP-based microarrays can also detect the parental origin of any chromosomal abnormalities. This may be valuable in rare instances of young couples producing many chromosome abnormalities but of little relevance to cases of advanced maternal age where at least 90% of the aneuploidies will be maternal in origin, and those of paternal origin are most likely mitotic errors where the paternal chromosome was randomly recruited as the extra chromosome [72]. These errors offer no predictive value for other embryos in the cohort or for future cycles; (b) SNP microarrays applied to PGD for chromosome rearrangements can differentiate between normal and balanced (carrier) embryos. However, because the rate of abnormalities in translocation cases is generally very high (>80%) [73], the great majority of PGD cycles do not have a surplus of embryos with a balanced chromosome constitution. In most cases, whatever balanced embryos are available are needed for transfer, (c) SNP arrays can directly produce a fingerprint of the embryo, allowing for assessment of which of the transferred embryos led to a pregnancy. With SET of blastocysts becoming the trend in PGS, this is of little use except to determine misdiagnoses. (d) Finally, qualitative SNP arrays can also detect uniparental disomy (UDP), although this is a very rare event (e.g., UDP 15 occurs in 0.001% of newborns (OMIM)).

A major disadvantage of a qualitative or combination approach to SNP array analysis is the need to assess parental DNA ahead of the PGD cycle. This complicates patient management, adds substantially to the cost of the test, and precludes ad hoc decisions on biopsy for PGD. Approximately 20% of IVF cycles with planned PGD are canceled on day 3 due to low embryo numbers. Thus, these patients would have spent money on pre-cycle parental testing that was ultimately unnecessary.

Table 49.1 Comparison of techniques

	% embryos	FISH	qPCR	aCGH	Embryo Vu	SNP array	hr-NGS
Labs performing test		100s	2	180	1	11	5
Total independent data signals*		11	96	2,700	26,000	32,000	700,000
Resolution in Mb		Arm	20M	6M	20M	6M	3M
Misdiagnosed aneuploidies (a-f)		7%	1%	2%	3% d	2%	0%
Unbalanced translocations (g)	2%	Custom	No	Yes	No	Yes	Yes
Partial aneuploidies	5%	No	No	Yes	Some	Yes	Yes
Polyploidy	2%	Yes	No	No	No	Yes	Yes
Mosaicism (h, i)	20%	20%	No	4%	No	No	20%
Miscarriage rate (j, k)		10–20%	20%	13%	Unk	Unk	11%

49.6.4 qPCR

The qPCR method was developed as a rapid, 4-h chromosome aneuploidy screening technique [74, 75] to enable comprehensive chromosome screening in fresh embryo transfers (FET). One of the key factors unique to this method is its independence from whole genome amplification methods. Ninety-six loci were identified and then amplified for this method. The threshold for amplification was representative of the amount of DNA template and hence the chromosome number in the sample. The significant advantage of this particular method also lies in the low amount of time required to perform the assay and complete the analysis. As we have discussed above, CGH-based methods are not suitable for fresh embryo transfers. The qPCR method for aneuploidy screening is an ingenious repurposing of a method traditionally used to perform gene expression studies, where relative transcript quantities are determined. Parental DNA is not required for this technique. qPCR for PGS is well validated and has promising results from CRTs [8, 76]. The major disadvantages of qPCR are the inability to detect structural abnormalities and the low resolution due to amplification of only four loci per chromosome (>20 Mb). Similarly, mosaics cannot be detected with this method.

49.6.5 High-Resolution NGS

Massively parallel genome sequencing, also known as next-generation sequencing (NGS), is the latest and currently the most popular technique being utilized in PGS laboratories throughout the USA. Compared to the older techniques, like the SNP array (32,000 reads) and aCGH (2700 reads), high-resolution NGS (hr-NGS), which is currently being used in the laboratory, derives about 700,000 reads per sample. hr-NGS utilizes similar upstream technology as aCGH and SNP arrays. Biopsy of the trophectoderm from the embryos is used as starting material. Whole genome amplification is performed on the biopsy. At this stage, the protocol for hr-NGS deviates from aCGH and SNP. One of the major advantages of NGS is the ability to scale up and run a large number of samples together by multiplex sequencing, thereby reducing the cost per sample for every procedure. Multiplexing is the ability to run multiple samples on a single run on the sequencing machine. NGS makes this a possibility by virtue of the addition of "barcodes." Barcodes are small (6–10 bp) sequences that are unique oligonucleotides that ligate to the ends of the fragments of the DNA (derived from the blastocyst day-5 biopsy) being sequenced. Barcodes help differentiate between different samples when sequenced. The possibility to scale up as a result of multiplexing is the first major deviation between the processing of samples for SNP/aCGH and hr-NGS.

In most cases, DNA sequencing unlike aCGH and SNP array isn't limited in coverage to the areas of the genome that are attached to the chip. The process of DNA sequencing involves processing one base at a time. Sequencing is performed on DNA that has been randomly amplified during WGA stage. These sequences are aligned and compared to a reference or a master sequence, which is a database of previously sequenced genomes. Post-alignment in hr-NGS, the sequenced fragments are then mapped back to each chromosome.

49.6.6 Targeted NGS

Another modification of the next-generation sequencing technique is the targeted NGS approach. This approach like the qPCR approach does not include the whole genome amplification step [77]. Instead scientifically defined pieces of the genome are amplified using the PCR method, and then the amplified product is sequenced. These strategies are usually lower in resolution due to selection of limited defined regions. However guaranteed coverage of the specified sequences (desirable genes and polymorphisms) enables detection of haploidy, triploidy, specific pre-selected microdeletion targets, uniparental disomy (UPD), and mitochondrial DNA quantification [77]. It is important to keep in mind that detection of microdeletions, UPD, and mitochondrial DNA is impending on the selection of the targets before sequencing. This is different from the hr-NGS technique where whole genome amplification is random in nature and prediction of the said abnormalities is inaccurate. Additionally, since each chromosome is examined at fewer sites, fewer reads are utilized which allows for more samples to be run simultaneously and in turn lower the cost per sample. Another advantage of the targeted approach is its ability to detect imbalances with chromosome rearrangements [77].

49.6.7 Validation ofPGD V2 Techniques

Due to the intrinsic and often unforeseen problems with every new technology, a novel method should always be validated against other more established methods [78]. Assessing a new approach against itself may preclude the detection of technique-related flaws. Validation by inadequate methods is possible and may lead to false assumptions when (1) the analysis of cell lines with defined chromosome abnormalities which cannot mimic mosaicism and other peculiarities of the cell being tested, (2) analysis of embryos by one technique and the remainder of the embryo by the same technique which precludes identifying abnormalities not detectable by that technique, (3) blindly replacing undiagnosed embryos (either by single embryo transfer or fingerprinting the embryo) and following pregnancies and clinical losses determine the fate of each tested embryo, which does not account for the status of non-implanted embryos. In addition, the use of analysis tools that are qualitative in nature will miss the presence of two chromosomes of the same grandparental origin, and the errors caused by mosaicism will not be taken into account in this validation mode, resulting in bogus 99.9% confidence results. In our opinion, the optimal method for validating any new technique is to reanalyze those embryos

that were not transferred to the patient. The reanalysis of these embryos should be done with another well-established technique, i.e., the "gold standard." This would discern shortcomings of the new method under evaluation and account for issues related to embryo biology, such as mosaicism. To simplify comparison between studies, an error should be classified as diagnosing an embryo as euploid when reanalysis shows that it was abnormal or vice versa. Due to the extent of mosaicism, an error rate per chromosome has questionable relevance and no clinical importance compared to an error rate per embryo. SNP microarrays have undergone a variety of validation experiments, such as comparison of PGD results and analysis of babies born [70], SNP microarray reanalysis of embryos previously analyzed by SNP arrays [79], and using data from one set of SNPs as internal controls for another set of SNPs. To date, no studies have confirmed the original diagnosis by reanalyzing the remaining embryonic cells with a different technique.

Microarray CGH for PGD has been validated by analysis of single cells from known cell lines (Dagan Wells, personal communication) and by analyzing eggs with aCGH and comparing them to the results obtained using aCGH of the corresponding PBs (Montag and Gianaroli, personal communication). Day-3 embryos analyzed by PGD with aCGH that were not replaced because of chromosome or morphological abnormalities were reanalyzed in most of their remaining cells by FISH using 12 probes for the most common chromosome abnormalities plus probes for any chromosomes found abnormal according to aCGH. Only 1.9% of embryos were found to be incorrectly diagnosed [67]. This is lower than the 7% error rate expected solely from mosaicism as calculated in FISH studies [10].

The qPCR technique validation is probably one of the most complete validation techniques for PGS technologies. Validation was performed in several phases. The first phase included randomized blinded samples from cell lines with previously determined karyotype by a gold standard g-banding method. An overall consistency of 99.90% (1007/1008) and an overall 24-chromosome diagnosis consistency of 97.60% (41/42) were observed in this phase [75]. No false negatives were observed. In the following phase, 71 embryos with reliable SNP microarray-based 24 chromosome aneuploidy screening results from double biopsies were biopsied again, randomized, and blinded for analysis. The validation of this technique in embryos had an overall chromosome-specific consistency of 99.94% (1703/1704) and an overall 24-chromosome diagnosis consistency of 98.6% (70/71). There were no false positive aneuploid chromosomes observed or inaccurate predictions of sex. Results from the cell line and the embryo phase were also found to be concurrent for the qPCR technique.

hr-NGS validation was recently performed by Fiorentino et al. in 2014 and Kung et al. in 2015 [80, 81]. In these studies, dependability of NGS-based 24-chromosome copy number assignments was gauged with previously established array-CGH–based diagnoses of the same WGA products at the level of individual chromosome copy numbers for the entire 24 chromosomes of each sample tested and also for the overall diagnosis of euploidy or aneuploidy. NGS specificity for an aneuploidy call (consistency of chromosome copy number assignment) was observed to be a 100% with a sensitivity of 100%. NGS specificity for an aneuploid embryo call (24-chromosome diagnosis consistency) was also a 100% with a sensitivity of 100%. An overall error rate of 0% was observed during the validation of NGS.

Table 49.2 RCT studies using PGS v2

Implantation rate				
	Technique	no PGT	PGT	
Yang et al. 2012	aCGH	46%	69%	$p < 0.001$
Scott et al. 2013	qPCR	63%	80%	$p < 0.001$
Forman et al. 2013	qPCR	40%	58%	$p < 0.001$
Rubio et al. 2017	aCGH	28%	53%	$p < 0.001$

49.6.8 Clinical Results

Initial CGH and aCGH studies [53, 82–84] were not randomized but showed improving ongoing pregnancy rates. Two studies [54] showed high implantation rates, avoided cleavage-stage embryo biopsy, and transferred embryos that had previously been cryopreserved in a later cycle. In addition to the potential benefits of transferring euploid embryos, there may be additional advantages associated with transfer in a non-stimulated cycle [85]. Loss of blastocyst-stage embryos after devitrification in the study by Schoolcraft et al. was minimal (0.7%) [53] . Blastocysts are more resilient than the day-3 embryo [86]. This resilience seems to increase the overall implantation rate by almost 20% when day-5 blastocyst biopsies are used instead of cleavage-stage biopsies. It is important to remember that despite the available data, biopsies are subject to differ greatly between operators, which in turn would alter the success rates greatly too.

The first randomized clinical trial (RCT) using PGD v2 was performed by [68] using aCGH in a group of young patients, showing a significant improvement in ongoing pregnancy rates. Two other RCTs using SNP arrays followed: one by [8] also showing significant improvement in ongoing pregnancy rates in a group of good prognosis patients and another RCT by [76] which showed that replacing one euploid blastocyst achieved the same ongoing pregnancy rate than replacing two untested embryos (Table 49.2). Two meta-analyses show that overall PGS v2 produces a significant improvement in ongoing pregnancy rates [87, 88]. Most recently, [5] published a RCT using aCGH combined with day-3 biopsy in AMA patients showing a significant improvement in ongoing pregnancy rates and showing that even with day-3 biopsy, if performed by a center with experience, it can produce an improvement in results.

49.6.9 Mosaicism

hr-NGS has an increased dynamic range which enables the detection of mosaicism in multicellular samples [80]. Chromosomal mosaicism is an established and relatively common phenomenon in the preimplantation embryo which has both diploid and aneuploid cells [89]. About 21% embryos are observed to be mosaic in the clinic [90]. When clinical losses of aCGH-analyzed euploid embryos were reanalyzed by hr-NGS, almost 50% of the cases ended up being mosaic [90–93] and showed that mosaic embryos have lower rates of implantation than euploid embryos but can implant as previously shown by [94]. So far, all babies born from embryos classified as mosaic at blastocyst stage have resulted in unaffected children (unpublished), and the current hypothesis is that these embryos will not implant or will miscarry depending on the load of abnormal cells, and if the embryo has enough normal ones, those will take over [49]. In humans, the same tendency seems true [90]. As proposed by [90, 95] and PGDIS, mosaic embryos have an intermediate pregnancy outcome potential between euploid and aneuploid and should be deprioritized for transfer but can be replaced if no euploid ones are available. Initial studies comparing aCGH to hr-NGS seem to indicate an advantage of the second over the first by not replacing mosaic embryos (Friedenthal et al. 2017, ESHRE).

49.6.10 Chromosome Abnormalities AreCenter-Dependent

Not all chromosome abnormalities are due to maternal age. We described a long time ago that sub-optimal culture conditions could produce more post-meiotic abnormalities [96]. In a recent study, we compared the rate of aneuploid embryos derived from egg donors from different fertility centers. The only variable that differed between these centers was the center itself, and we detected rates of aneuploidy that ranged from 20% to 60% [97]. These differences could be attributed to differences in stimulation protocol from one IVF lab to another, media differences from one manufacturer to another-temperature and pH could also potentially be affecting the final outcomes-or which follicles are retrieved (i.e., small and immature ones may result in more abnormal embryos). Such variables need to be further controlled in future studies to improve IVF techniques and therefore PGS can be used as quality control [98].

In summary, data on the clinical application of comprehensive chromosome analysis techniques suggests a better prognosis for patients undergoing ART with increased implantation rates, on-going pregnancy rates, and decreased miscarriage rates.

Review Questions

1. More than _____ in vitro embryos are found to be chromosomally abnormal.
   A. 10%
   B. 25%
   C. **50%**
   D. 35%

2. Embryos from which of the developmental stages may be used in obtaining biological material for genetic testing of embryos?
   A. Polar body biopsy
   B. Blastocyst biopsy
   C. Blastomere biopsy
   D. **All of the above**

3. Multiplexing of a large number of samples is possible in next-generation sequencing. This is possible due to
   A. **Addition of barcodes, 6–10 bp unique oligonucleotides**
   B. High number of reads possible during a sequencing run
   C. Blastocyst biopsy samples being used for NGS compared to polar body or blastomere biopsy samples
   D. None of the above

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Embryo Biopsy for PGD: Current Perspective

Steven J. McArthur, Don Leigh, Maria Traversa, James Marshall, and Robert P. S. Jansen

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Learning Objectives

- To describe the rationale for blastocyst-stage biopsy in comparison to biopsies performed at earlier embryo developmental stage.
- To review the settings where blastocyst-stage embryo biopsy can be performed.
- To provide a detailed description on how blastocyst-stage embryo biopsy is performed.
- To present the applications of blastocyst-stage biopsy.
- To review the objectives and means of single gene testings using embryonic cells.

This chapter sets out to examine the major recent advances in embryo biopsy, specifically blastocyst-stage biopsy and preimplantation genetic testing and diagnosis (*PGD*), which are transforming singleton live birth rates in families at risk of passing on monogenic diseases or chromosomal translocations.

50.1 The Development of Embryo Biopsy

Embryo biopsies for clinical PGD generally were performed on day 3, when the embryo typically was at the six- to eight-cell stage, and involved the removal of one or two blastomeres. The zona was breached by dissolving the protein with acid Tyrode's solution. The embryo was incubated in a calcium-/magnesium-free medium to reduce cell–cell interactions and make the removal of cells easier. There has been debate about the impact of removing multiple cells, and in general, it was considered that two-cell removal was more detrimental than removing just a single cell and should not be performed—although the reliability of amplification of two cells was considered less prone to allele dropout. An alternative biopsy approach involved the removal of either the first or second polar bodies—either sequentially on day 0 and day 1 or both on day 1. PCR analysis of blastomeres or polar bodies involves amplification of a single allelic copy of the target. Similarly, analysis by FISH is a single-cell test. Biopsy at the blastocyst stage, when embryos typically comprise upward of 100 cells, enables the removal of 3–5 trophectoderm cells without significant cell mass depletion. Putting several cells into a PCR reaction should decrease the likelihood of amplification failure (allele dropout, or ADO) and, with FISH analyses, should provide an opportunity to confirm signal patterns.

During the early 2000s, Genea (formerly Sydney IVF) moved comprehensively to blastocyst culture and blastocyst-stage transfers and cryostorage and experienced a corresponding increase in take-home baby rates, substantial reductions in multiple pregnancies, and reduced rates of miscarriage [1–7]. In 2004, we described the first clinical application of blastocyst biopsy to routine PGD practice [1]. Embryos were "hatched" on day 3 using a Hamilton Thorne ZILOS-tk near-infrared laser and were then incubated for another 2 days to enable blastocoel expansion and herniation of trophectoderm cells through the opened zona for biopsy [1, 6]. Suitable embryos were placed in 5 μL drops of standard medium under oil. A holding pipette, the same as used in ICSI practice, was employed to immobilize the embryo, while a 30-μL biopsy pipette was used to collapse the blastocyst cavity and hold the tissue sample. Several pulses with the laser set at low level loosened cell–cell interactions, permitting a small piece of tissue to be teased off the exposed trophectoderm. The embryo was then removed and placed into fresh medium for further incubation (until the results were known and the embryo was transferred, cryostored, or disposed of). The tissue piece was washed and placed into PCR tubes or fixed to glass slides for analysis using FISH.

The advantage of moving from cleavage-stage to blastocyst-stage PGD was demonstrated by comparison of embryo biopsies performed (a) at the day 3 cleavage stage and followed by the transfer of embryos that went on to blastulate successfully, with (b) embryo biopsies taken *at* the blastocyst stage (day 5–6) and followed by almost immediate transfer [7]. This study, in other words, *examined the efficacy of day 3 biopsy* vs. *day 5 or 6 on the embryo while controlling for the embryo's ability to blastulate*; patients in this trial had PGD not for infertility or miscarriages but to prevent further propagation of a serious monogenic family disease. The outcome (Table 50.1) implies that, in comparison with the biopsy of blastocysts, day 3 cleavage-stage PGD reduces the implantation potential of at least some embryos. (The same could still also be true of blastocyst biopsy but appears to be to a much lesser extent.)

Table 50.1 shows the technical outcome data for the embryos biopsied (595 for day 3; 656 for days 5–6), with an

Table 50.1 Embryos available for testing for monogenic disease mutations by biopsy on day 3[a] and on day 5–6

	Egg retrievals	Embryos biopsied	Inconclusive test result	Conclusive, favorable test	Embryos transferred fresh	Tested embryos cryostored
Day 3 biopsy + day 5–6 transfer[a]	91	595 av. 6.5 embryos	61 (10.3%)	261 (43.8%)	103	158 (60.5%)
Day 5–6 biopsy + day 5–6 transfer	177	655 av. 3.7 embryos	46 (7.0%)	305 (46.5%)	121	184 (60.3%)

See McArthur et al. [7] for more detailed interpretation of the data
[a]All embryos were developed to blastocysts before transfer

Table 50.2 Clinical outcomes following biopsy at the cleavage stage vs. biopsy at the stage of blastocyst, each with transfer of embryos fresh on day 5 or 6

	Embryos transferred *n*	Transfer procedures *n*	Pregnancy per retrieval	Implantation per embryo[a]	Miscarriage	Live birth or ongoing pregnancy	Multiple at confinement		
							Single	Twin	Triple
Day 3 biopsy + day 5–6 transferred *n* = 91 retrievals	1	38	11	11	4	7	7		
	2	28	12	15	1	10	7	2	1
	3	3	1	1	0	1	1		
	All av. 1.5	69 (75.8%)	24/91 (26.4%)	27/103 (26.2%)	5/91 (20.1%)	18/91 (19.8%)	15	3 multiples (16.7%)	
Day 5–6 biopsy + day 5–6 transferred *n* = 177 retrievals	1	105	54	54	8	46	46		
	2	8	4	5	1	3	2	1	
	3	0							
	All av. 1.1	113 (63.8%)	58/177 (32.8%)	59/121 (48.8%)	9/58 (15.5%)	49/177 (27.7%)	48	1 multiple (2%)	

Data from McArthur et al. [7]
[a]The implantation rate for blastocysts biopsied from the trophectoderm as blastocysts (48.8%) was highly significantly better than the implantation rate for blastocysts biopsied using a single cell removed at the day 3 cleavage stage (26.2%, $P < 0.01$)

average of 6.5 embryos biopsied and tested per retrieval at cleavage, compared with an average of 3.7 embryos biopsied and tested per retrieval when the blastocyst stage was awaited before performing PGD. The proportion of embryos with a conclusive test and with a normal result, thus suitable for transfer, was still approximately 50% in each series, which means that taking the biopsy later in embryo development conferred appreciable laboratory and clinical efficiency through not having to test embryos whose development was compromised. The late-biopsied blastocysts had almost twice the chance of implanting than did the blastocysts that had been biopsied on day 3.

Table 50.2 shows the outcomes of the embryo transfer procedures. In spite of a lower average number of embryos transferred (1.1 vs. 1.5 per transfer procedure), and without taking into account later further pregnancies from cryostored, biopsied embryos, the day 5–6 biopsy transfers resulted in fewer miscarriages and a higher absolute ongoing pregnancy rate, as well as the expected lower rate of multiple pregnancy. There was one obvious monozygotic twinning event, involving an embryo biopsied on day 3. In about 60% of cases in each series, additional embryos that had tested normally were cryostored for further attempts at pregnancy.

50.2 Preimplantation Screening for Aneuploidy

It has been known for more than 15 years that IVF embryos show a high rate of chromosome aneuploidy [8]. It has also been understood for many more years that a chance acquisition of an abnormal number of chromosomes is a frequent event in human conception and, in particular, is the commonest cause for pregnancies to miscarry. It might therefore be expected that screening IVF embryos for aneuploidies before selecting an embryo to transfer should materially improve the chance of pregnancy, reduce the risk of miscarriage, and (by enabling embryos to be transferred efficiently and efficaciously one at a time) greatly reduce the multiple pregnancy rate, thus lessening perinatal morbidity and mortality. The target—improved live birth rates from IVF and less costs for community—is worthy and logical.

50.3 Aneuploidy Risk

Several authors have reported that the age of the woman undergoing IVF has a significant bearing on the extent of aneuploidy in the resulting embryos. Studies on the origin of nondisjunction chromosome anomalies have suggested that most of the abnormalities originate predominantly from female meiosis, especially meiosis I, although analysis of preimplantation embryo polar bodies with FISH has indicated that meiosis II errors could be similar in number [9]. Analysis of later-stage embryos would therefore be able to identify both meiosis I and meiosis II errors (as well as reveal aneuploidies brought by the fertilizing sperm). Generally, a predisposition to aneuploidy beyond maternal age effect has been hampered by the fact that few studies have looked for or been able to identify genetic causes; rare recessive genetic states that interfere with meiosis have been described [10]. While not extensive, there have been a number of reports suggesting that among women undergoing IVF and experiencing subsequent implantation failure, the chromosome

abnormality rate in their embryos is quite high compared to the other IVF cohorts [11–13]. Screening embryos for aneuploidy could reduce the number of embryos subsequently needed to initiate a successful and continuing pregnancy [14]. The efficacy of the screening process must obviously take into account any detrimental aspects of the biopsy and culture processes to be considered truly beneficial for the patient's progress.

50.4 Aneuploidy Screening in IVF Programs

Examination of a restricted number of chromosomes using FISH for aneuploidy screening as a routine may not be helpful in all cases and in fact can be harmful if biopsy procedures are not efficient. Mastenbroek et al. showed that biopsy of day 3 (cleavage-stage) embryos for limited PGS—screening for aneuploidy of chromosomes 13, 16, 17, 18, 21, X, and Y—can reduce the chance of an ongoing pregnancy in women aged 35–41 having in vitro fertilization (IVF) [15]. Our considerations above (Tables 50.1 and 50.2) suggest that interfering with an early embryo might lie behind this detrimental result, but other factors could also be important. For the Mastenbroek study, these included such straightforward concerns as the time the embryos spent being manipulated in potentially altered culture conditions across the variety of IVF clinics where the biopsies were performed. They also include more complex issues, such as the inadvertent exclusion from transfer of mosaic embryos in which the biopsied cell happened to be the only cell with trisomy (a situation that can follow a mitotic nondisjunction event) [9].

Between August 2004 and November 2006, we studied the impact of screening for aneuploidy in younger infertile women (<38 years, median 33.5 years), employing biopsies of blastocysts [5]. All women were in their first or second attempt at IVF. Agreement to have one embryo transferred (eSET) was a precondition for entry. Patients were withdrawn from the study if there were fewer than eight ovarian follicles over 1 cm diameter at 8–10 days of stimulation, fewer than four embryos with seven or more cells on day 3 of culture, or fewer than three blastocysts for biopsy on day 5 or 6; no women had cycles canceled because of a poor response. The biopsies consisted of 2–9 trophectoderm cells and were tested by at least five-color fluorescent in situ hybridization for, at minimum, chromosomes 13, 18, 21, X, and Y. We compared outcomes between the screened group (Group A, normal [3]five-color pattern in all the removed trophectoderm cells for the transferred embryo) and the principal control group (Group B, with zona opening but no biopsy); we also made comparisons with the women who were withdrawn from the study before randomization because of suboptimal responses to stimulation (Group C) and with women who were eligible but elected not to take part in the study (Group D). Table 50.3 gives the results up to the time the trial was suspended. Pregnancies are clinical pregnancies with a normal fetal heart rate on ultrasound scanning in the first trimester. The clinical pregnancy rate (pregnancies with a normal fetal heart rate at 6 weeks' gestation) was high (46.4% of egg retrieval procedures overall), irrespective of whether PGS was performed or not, and is consistent with results we [2, 6] and others [16] have reported previously for elective single blastocyst transfers.

Table 50.3 Pregnancy rates after preimplantation genetic screening for aneuploidy from biopsy of blastocysts on day 5 or 6 of development using five- or seven-color FISH

Group A. Biopsy				Group B. No biopsy (control)			
N	P	NP	%P	*n*	P	NP	%P
56	25	30	45.5%	48	26	20	56.5%
Group C. Poor response, withdrawn				Group D. Eligible, nonparticipating			
N	P	NP	%P	*n*	P	NP	%P
107	36	71	33.6%	1194	564	630	47.2%

From Jansen et al. [5], with permission
P pregnant; *NP* not pregnant

Among the women who underwent biopsy for aneuploidy screening (Group A), the pregnancy rate at 45.5% was insignificantly less than among women who were eligible for the trial but did not take part (Group D, 47.2%) and was trending to be higher than among women who were withdrawn from the trial prior to randomization because of a suboptimal response (Group C, 33.6%; $c^2 = 1.7$, $P < 0.1$, 1-tailed). We could thus find no evidence of clinically important detriment from blastocyst biopsy in women of normal reproductive age. The pregnancy rate compares favorably, with the 25% clinical pregnancy rate reported by Mastenbroek et al.

Unexpectedly, Group B, the embryos subjected to zona opening by near-infrared laser, a standard preparatory step for biopsy and performed on day 3 or 4 (see above), produced the highest clinical pregnancy rate of the groups (56.5%). While the results in Group B were not statistically significantly different from either the biopsied embryos (Group A, $c^2 = 0.8$) or the eligible but nonparticipant women's embryos (Group D, $c^2 = 1.2$), the trend was opposite to that required to disprove the null hypothesis, and the clinical trial was stopped.

The reason for the strong performance of the embryos in the principal control group, if it is true, is not clear. Assisted hatching by opening of the zona, while advocated from time to time for the embryos of older women to facilitate hatching and implantation, has not been shown to be beneficial among women under 40 or with good blastocyst development. More likely, a too strict set of criteria for assumed meiotic nondisjunction led to overinterpretation and rejection of some blastocysts that would, if left unscreened, have developed normally and contributed to the total number of embryos suitable for transfer.

50.5 Testing for Chromosomal Translocations

Reciprocal translocations occur in about 1 in 625 newborns and usually result from the exchange of two terminal segments from different chromosomes, ordinarily resulting in a genome that is balanced. Exchanges can also take place close to the centromeres of two acrocentric chromosomes; these Robertsonian translocations, which occur in about 1 in 900 newborns, also ordinarily provide a balanced genome and bring the overall prevalence of balanced translocations among newborns to about 1:380 [17]. When diploid germ cells with these karyotypes eventually undergo meiosis, however, the chromosomes involved segregate abnormally and yield a varying but significantly high level of unbalanced haploid states among oocytes and spermatozoa—an unbalanced state that is continued into the embryo and which results in implantation failure, miscarriage, stillbirth, or abnormalities at birth. Balanced translocations are ten times more common among couples presenting for treatment with IVF [18].

With *reciprocal translocations*, homologous pairing during meiosis 1 produces a tetravalent structure instead of the usual bivalent. Subsequent segregation to respective daughter cell spindles takes one of three modes: *2:2 alternate segregation* (producing alternately a normal or a balanced abnormal complement, the latter perpetuating the familial condition but both with a balanced genome); *adjacent 1 and 2 segregations* (producing segmental monosomies and trisomies); and, comparatively rarely, 3:1 *segregations* (involving nondisjunction of a whole chromosome and producing more complete monosomies and trisomies) [19]. Overall, 75% of embryos from a parent with a balanced reciprocal translocation show partially or fully aneuploid chromosome complements (14 different unbalanced combinations compared to two balanced combinations), considerably reducing the number of otherwise healthy appearing embryos available for transfer after PGD.

In *Robertsonian translocations*, a trivalent structure is formed during meiosis 1, with three main segregation modes possible (and nine different chromosome combinations), namely, *alternate* (which returns dosage to its balanced state), *2:1 segregations* (producing complementary monosomies and trisomies), and *3:0 segregation* (producing double trisomy or double monosomy).

Traditional PGD for translocations involves FISH, utilizing either breakpoint-spanning probes (which require access to extensive probe libraries and complicated workups) or (much more simply) combinations of commercially available, quality-controlled centromeric, locus-specific, and subtelomeric probes attached to standard fluorochromes. The use of PGD to screen balanced from unbalanced chromosome sets in the embryos then significantly reduces the failure rate for implantation and should result in fewer miscarriages among the embryos available for transfer [13, 20]. Again, to be truly beneficial, the process of biopsy must do the least amount of harm to the embryo's continued development and to its ability to implant. Table 50.4 shows our experience with cleavage- and blastocyst-stage biopsies among couples with recurrent miscarriage attributable to a balanced reciprocal translocation in one of them. The live baby results have been lower compared to those we obtain after testing for monogenic disease (Table 50.2), possibly reflecting the large decrement in transferable embryos seen with reciprocal translocations following the demonstration of unbalanced cells by FISH-based PGD. These apparent unbalanced outcomes can be of biological origin but can also be false, due to inherent error rates observed with FISH-based protocols [21] or reflective of a benign mosaic state,

Table 50.4 Clinical outcomes of PGD for balanced translocation using FISH and from using STR-based PCR

	Reciprocal translocations		Robertsonian translocations	
	FISH	STR-PCR	FISH	STR-PCR
Patients	54	22	17	7
Mean age	35.2	33.1	36.0	33.3
Cycles with egg retrieval	112	22	51	6
Cycles with embryo biopsy	73	22	31	6
Biopsied embryos	320	61	142	17
Actionable PGD result	304	60	136	17
Embryos for transfer	74 (23%)	20 (33%)	44 (31%)	13 (76%)
Embryo transfer cycles	54	13	27	5
Embryos transferred	1.2	1	1.2	1
Total embryos transferred	62	13	32	5
Positive pregnancy test	20	7	14	3
Implantation rate/embryo (%)	32	54	44	60
FH-positive pregnancy rate per transfer	15 (24%)	6 (46%)	12 (38%)	2 (40%)
FH-positive pregnancy rate per egg retrieval (%)	13	27	24	33

Data from McArthur et al. [7] and Traversa et al. [22]
All embryos were biopsied and transferred at the blastocyst stage. Results do not include pregnancies from the embryos cryostored

but in either case contributing to false-positive interpretation of FISH signals and leading to the exclusion of otherwise normal embryos.

In our published series for translocations using FISH [7], 95 egg retrievals were performed and led to biopsy and testing among couples with a *balanced reciprocal translocation*; there were 10 pregnancies among 26 patients who had day 3 biopsies, seven of which went to term—a miscarriage rate of 33%. Of the 12 pregnancies among 21 couples for whom biopsy was performed on day 5–6, eight miscarried (38%). Twenty-three egg retrievals among 15 couples with a *Robertsonian translocation* led to biopsies and testing; there were three pregnancies among seven couples with day 3 biopsies, each of which went to term, and eight pregnancies among seven couples with day 5–6 biopsies, one of which miscarried and one of which was an ongoing monozygotic twin pregnancy. Combining day 3 with day 5–6 biopsies, the miscarriage rate after PGD for Robertsonian translocation exclusion was 18%, whereas PGD for excluding unbalanced reciprocal translocations was followed by a miscarriage rate of 45%.

50.6 Monogenic Diseases

Monogenic diseases considered appropriate for PGD are those uncommon or rare, fatal, or chronically disabling familial conditions that occur as a result of mutations in a single gene. The location of the mutation can be in an exon, a splice point, or within the control regions and affects the functioning of the specific gene. Inheritance is Mendelian, and classically there are three major classes of phenotypic expression:

1. *Dominant inheritance*, where every individual who inherits the single gene change is likely to be affected by the disorder and will carry a 50% chance of passing on the affected gene to offspring. An example is Huntington's disease. PGD analysis for such mutations must be reliable in detecting a mutation change in a background of normal DNA sequence.
2. *Recessive inheritance*, where carriers of mutations themselves are not affected by the disorder but who partner with another carrier for a mutation in the same gene then produce a reproductive risk for their offspring of 25% for an affected child and 50% for a carrier child. An example is cystic fibrosis. Mutation analysis for these conditions needs to address the ability to analyze for a mutation in a homozygote state or often in a compound heterozygous state.
3. *X-linked inheritance* where, essentially, mutations on the X chromosome typically result in female carriers who have a 25% risk of producing affected male offspring and a 25% risk of reproducing the carrier state in female offspring. An example of a recessive X-linked gene disorder is hemophilia A. An example of an incompletely dominant X-linked disorder is fragile X syndrome, which causes severe mental retardation in males but which also has a heterozygous female phenotype that includes premature ovarian failure. Analysis must be reliable but, unlike other recessive diseases or the dominant diseases, there is no normal background DNA sequence for males. Female carriers contribute a nonmutated X chromosome, so confidence with the analysis must be the same as for the autosome mutations.

The starting point for PCR in the case of a single cell from a day 3 biopsy is usually just a single copy of DNA (there is obviously more DNA available with multicellular trophectoderm biopsies). In principle—and regrettably sometimes also in practice—failure of the mutated DNA to amplify (ADO) produces a false-negative result, leading to an incorrect conclusion of a normal state. Any biopsy testing process must be as reliable as possible to avoid any miscalls.

50.7 The Near Future for Translocation Testing

50.7.1 STR-Based Molecular Strategies

Our experience, above, revealed no obvious advantage for blastocyst-stage biopsies compared with day 3 cleavage-stage biopsies when FISH is used to infer balanced chromosomal patterns for either reciprocal or Robertsonian translocations. In each case, miscarriage rates remain particularly high for apparently balanced reciprocal translations. We have since reported a molecular strategy utilizing PCR for PGD in translocation carriers that examines highly polymorphic short tandem repeat sequences (STRs), application of which has significantly improved outcomes after biopsies at the blastocyst stage [22].

Using STR profiling to identify chromosomal segments on either side of the known breakpoints, in conjunction with standard cytogenetic segregation tables to predict each unbalanced state, we directly identify the monoallelic and triallelic states that are the direct cause of the phenotypic abnormality and reproductive loss which results from these malsegregants and, in turn, is the immediate pathogenic mechanism behind the reason PGD is offered to translocation carriers. The method requires extensive screening of chromosome-specific STRs to define those markers for which the carrier is heterozygous and where alleles are not shared with the partner. To make this PCR-based test efficient, chosen markers are multiplexed to obtain results within primary or secondary amplifications. The method also lends itself to other PCR-based PGD objectives conducted simultaneously, such as monogenic disease exclusion. Verification of the method has come from the rebiopsy of embryos diagnosed as unbalanced: in each of six cases in which samples were assessed for segmental chromosomal gains and losses using conventional CGH (see below), the predicted malsegregations were confirmed.

Conclusive results in our hands rose to 99% using STR profiling, compared with 93% with blastocyst-based FISH.

Any apparent mosaicism seen in the trophectoderm sample has the potential to complicate the interpretation of the translocation state, especially when using FISH, where any visible abnormality tends to disqualify the embryo for transfer, on the subjective basis that failure of a chromosome to hybridize or to hybridize ambiguously is always possible. STR profiling, on the other hand, encompasses multiple loci on each side of the translocation point, reducing ADO-based errors (false monoallelic states from diploid alleles, false biallelic states for trisomic alleles).

Table 50.4 compares our FISH-based blastocyst biopsy experience with our STR-PCR experience. Patients with reciprocal translocations still show the expected predominantly unbalanced segregation patterns predicted by theory, but fewer embryos are falsely disqualified from transfer. Patients with Robertsonian translocations also fare better. Robertsonian translocation carriers can be prone to uniparental disomy, especially when chromosomes 14 and 15 are involved (see [22]); STR-PCR, unlike FISH, enables biparental inheritance to be looked for and to be confirmed or excluded. Finally, the time needed for actionable results with STR-PCR is just 4–5 h, compared with the 6–16 h required for FISH hybridization and interpretation.

50.8 The Near Future for Aneuploidy Screening

The majority of aneuploidies arise during female meiosis. The minority are brought to the embryonic genome by the fertilizing sperm and are equally pathogenic. A small number take origin in the first few cleavage divisions through mitotic nondisjunction. The latter lead to mosaic states in the embryo: clearly, the later this happens, the smaller the proportion of triploid cells and the more patchy the distribution among inner cell mass and trophectoderm derivatives. There is a large body of published knowledge on the recognized outcomes, such as confined placental mosaicism. In the embryo proper, trisomic cells will be at a disadvantage compared with their euploid neighbors as tissues and organs develop. Our experience with karyotyping 82 cell lines derived from inner cell masses of slow and stalled embryos, assumed to disproportionally display aneuploidies, provides an indication of this process (Bradley et al., manuscript under review). Sixty-nine (84%) displayed only a normal, diploid karyotype, indicating likely self-correction of mitotic nondisjunction-based mosaic states; a limited number tested showed no cases of loss of heterozygosity, which would indicate uniparental disomy as a consequence of self-correction of meiotic errors. The 13 cellular outgrowths that were cytogenetically abnormal included six single trisomies, a double trisomy, a monosomy, three triploidies, a triploidy with an additional chromosome 22, and a balanced reciprocal translocation. In each of the trisomies, meiotic nondisjunction was confirmed by demonstrating triallelic states for STRs on the affected chromosome. There were no mosaic cell lines.

Thus, for reasons of both relative numbers (mitotic trisomies are from the start mosaic states, whereas meiotic trisomies are pure) and, possibly, a qualitative difference between independent aneuploid states compared with diploid states, the key objective of screening for aneuploidy should be less to count chromosomes than it is to recognize dominant original parent of origin states for any of the 24 chromosomes.

50.9 Fluorescent In Situ Hybridization

Specific staining of embryo chromosomes with FISH has been the preferred method to identify the chromosome copy number in a fixed cell preparation. The probes bind to defined regions on the cell chromosomes immobilized on a standard microscope slide, usually at interphase. Generally, the probes are purified cloned regions of the specific chromosome, subtracted for repetitive sequences. These probes are labeled with a unique fluorophore, which can be visualized with fluorescence microscopy. The preparation and quality control of such material generally means that a commercial supply of the probes is the preferred choice for routine clinical use.

There are limitations to commercially available probe sets. The number of fluorophore colors falls far short of the minimum of 24 required. The fluor needs to be chemically active to attach to the DNA probe and also stable enough to remain attached during the hybridization process. Once hybridized, the color must be able to be visualized using, typically, UV excitation and filtered emission. High-energy wavelength excitation can result in rapid photo bleaching of the fluorophore and hence insufficient time to enumerate the hybridization pattern. The commercial suppliers have limited their probe labels to a very small set that meet manufacturing standards and the exacting requirements for clinical use. These fluors must be spectrally separable using specific but simple microscope filters. In practice, this means that only 5–7 or so chromosomes can be checked in one hybridization event. Consequently, the number of chromosomes that are there to be counted using FISH means that multiple cycles of hybridization, enumeration, probe stripping, and rehybridization are needed. Each cycle runs the risk of target loss and/or degraded target sites, either of which can result in incorrect chromosome enumeration and thus a misreading of chromosome number—technical considerations that preclude more than two or three rounds of hybridization. Temporally, adding more than a very few hybridizations would take too much time to permit the transfer of IVF embryos fresh.

Single blastomere biopsy from day 3 embryos gives a single, simple answer: a normal chromosome complement or an abnormal complement. The problems of mosaicism and technical difficulties discussed above, however, still lead to embryos being incorrectly classified and then being excluded from transfer. Biopsy at the blastocyst stage does not resolve these problems but does offer an opportunity to see multiple

hybridization signals for a set of cells. Nonetheless, a conservative reading of those signals has meant that observation of mosaic states in multicell biopsies has resulted in the exclusion of embryos that are likely to be substantially normal and suitable for transfer. The policy of disqualifying an embryo for transfer on the basis of one or two aberrant cells might need to be reexamined.

All other current karyotyping methods applicable to extremely low copy numbers of chromosomes, including analyses of single cells with day 3 embryo biopsies and of typically fewer than ten cells with blastocyst trophectoderm biopsies, require preliminary amplification of DNA copy number.

The first way to satisfy this challenge is to greatly increase the number of chromosome targets to be amplified, enabling any genomic shortcomings in genome-wide amplifications to be overcome by averaging. Over the last few (very few) years, advances in whole genome amplification has advanced the place of the technique of *comparative genomic hybridization* (CGH) by increasing its resolving power within chromosomes as well as improving its quantitative reliability in estimating preamplification DNA copy number.

50.10 Comparative Genomic Hybridization

Developed as a chromosomal screen to analyze genomic changes in cancers almost 20 years ago [23], CGH reveals copy amounts of all 22 autosomes and the 2 sex chromosomes to a resolution of 10 million base pairs or so. The technique uses a combination of molecular and cytogenetic approaches to evaluate chromosome complements. Testing cancers with CGH is simpler than testing embryos, however, because generally with cancer samples there is no shortage of extracted DNA to be tested, whereas embryo biopsy specimens are much more limited.

Wells and Delhanty reported CGH analysis of individual cells from human day 3 embryos a decade ago [24]. Wilton and others reported the first successful clinical preimplantation use of CGH technique a year later [25]. While the use of CGH promised to deliver a total chromosome aneuploidy screen and the possibility of identifying any chromosome imbalance in an embryo, its labor intensity and its time-consuming nature (which required the embryos to be frozen while testing proceeded over periods of many days) precluded transfer of embryos during the biopsy cycle. There were only a few further reports over the ensuing 6 years [26–28]. Often, what was observed were relatively complex chromosome combinations; these then were given causal roles to explain implantation failure, but screening out aneuploid embryos did not improve embryo implantation rates. In spite of its promise, CGH has not been reported to be in routine use by any group. Recently, however, a report from Wells et al. has reported high implantation rates for blastocysts biopsied and analyzed with CGH after improved whole genome amplification [29]. The embryos were transferred after vitrification and later thawing and produced an impressive thawed blastocyst implantation rate of 67%—which would warrant routine use, at least in selected patients.

The use of classical CGH on metaphase chromosomes demands high levels of skill, many days of analysis, and the freezing of biopsied embryos until the karyotype is known. One approach to minimizing labor requirements and shortening the testing time has been to employ DNA microarrays [30]. On the one hand, the timing suits polar body analyses and day 3 cleavage-stage biopsies, but both of these sample types offer only a single-cell genome for amplification and analysis, whereas blastocyst biopsy offers several cells to average out the amplification biases more effectively. On the other hand, the additional expense of CGH, however performed, is coming to be more generally appreciated as another reason for identifying embryos that can blastulate before biopsy and testing, in effect providing a self-screening process that reduces the costs of the testing for the individual patient. A pilot study looking at the analysis of polar bodies for aneuploid detection of female origin has been commenced by a consortium from the European Society of Human Reproduction and Embryology. Implantation rates and pregnancy outcome data are still to be collected. Recent advances combining blastocyst-stage biopsy, micro-array CGH, and vitrification have produced high embryo implantation rates and clinical pregnancy outcomes allowing a viable clinical service to be offered [31].

To date, therefore, it is blastocyst-stage biopsy that has given valuable improvement in implantation rates, and it waits to be seen whether the still prevalent day 3 biopsies and day 0–1 biopsies of polar bodies can achieve the same outcomes. With the use of a DNA amplification-based approach, the interpretation problems associated with low-level somatic mosaicism common in embryos and seen with FISH are partially overcome. The tissue sample, typically consisting of 3–5 cells, is analyzed as a whole (and is taken to represent the embryo as a whole), thus producing an averaging effect for the constitutional chromosomes under investigation.

Review Questions

1. Please define the rationale of blastocyst-stage embryo biopsy.
2. Please describe the required settings for blastocyst-stage embryo biopsy.
3. Please explain the procedure of blastocyst biopsy and the removal of trophectoderm cells.
4. Please describe the general principles of single gene mutation testing (PGD or PGT-M) and the reasons for performing it.

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Cryopreservation

Contents

Sperm Cryopreservation

Ana D. Martins, Ashok Agarwal, and Ralf Henkel

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Learning Objectives

- To understand the indications, principles, and significance of sperm cryopreservation.
- To provide knowledge of different cryoprotectants and understand the mechanism of action, as well as the advantages and disadvantages of different categories of cryoprotectants.
- To provide basic knowledge of the gradual decrease in the temperature of the sample for the conventional slow cryopreservation method.
- To discuss the changes in sperm parameters after thawing.
- To discuss the ART outcomes in the use of cryopreserved spermatozoa.

51.1 Introduction

The term cryopreservation is derived from the Greek word κρύος (krýos, "icy cold, chill, frost"). This technique is used to preserve structurally intact living cells and tissues using very low temperatures. The origin of cryopreservation dates back to 1776, when Spallanzani froze sperm in snow and demonstrated their motility after rewarming. One century later, Mantegazza reported the survival of human sperm frozen at −17 °C for more than 4 days (for review [1]). In 1949, with the discovery of glycerol as a cryoprotective agent [2], it was possible to freeze viable spermatozoa for longer periods. In 1957, the first piglet was born from frozen-thawed porcine sperm [3]. In the 1970s, cryopreservation progressed significantly when freezing in dry ice and liquid nitrogen vapor was introduced [4]. However, the success of cryopreservation was achieved with the introduction of controlled rate freezing.

Following the first attempts in 1964, successful cryopreservation of human spermatozoa was then reported in 1973 for the first time [5]. Sperm cryopreservation, when properly performed, allows long-term storage in a state of metabolic arrest that prevents cellular aging while maintaining viability and fertilizing potential and is a critical part of assisted reproductive technology (ART) as it allows these spermatozoa to be used when needed. This process can be divided into three steps: (i) collection of the sample, (ii) freezing, and (iii) long-term storage in liquid nitrogen (LN_2) at extremely low temperatures of −196 °C.

The creation and maintenance of sperm cryobanks is regulated by the World Health Organization (WHO) [6]. Preservation of fertility is one of the main goals of sperm cryopreservation and, in some cases, the only opportunity for some couples to have children in the future. Consequently, it is also considered prior to any medical procedure that may potentially affect the fertility of the individual, for example, in some cases of malignant or non-malignant diseases. Cryopreservation is recommended for sperm donors or, in cases of absent partner, traveling husbands or men serving in the military. Moreover, freezing of sperm can be used to avoid the stress of multiple testicular or epididymal sperm extraction, as well as semen collection in cases of fertility treatment, thus avoiding the necessity of synchronization with female ovulation. Finally, this technique is recommended in cases where fertility may be compromised through medical interventions such as vasectomy and gender selection. In cases of pediatric illness, testicular tissue can be preserved. However, the use of testicular tissue for fertilization purposes still requires more optimization.

51.2 Principles of Cryopreservation

51.2.1 The Cryoprotectants

The cryoprotective effects of glycerol were accidentally discovered in 1948 [2]. Eventually, this discovery, and later on the discovery of dimethyl sulfoxide [7], led to the cryopreservation of many different types of cells. For the cryopreservation of spermatozoa, scientists, in 1952, reported the use of a hen egg yolk-sodium citrate buffer equilibrated with glycerol [8]. Besides glycerol and dimethyl sulfoxide, ethylene glycol and 1,2-propanediol have also been used as cryoprotectants.

Cryoprotectants protect cells, including spermatozoa, from the damage caused by ice crystal formation during the freezing process by decreasing the freezing point and thereby reducing the amount of salts and solutes [9]. On the other hand, such cryoprotective substances can have toxic properties, and it is therefore essential to understand not only their possible toxic effects but also the efficiency of the freezing process and the effect of cryoprotectant on spermatozoa. Thus, the success of cryoprotectants will depend on factors such as concentration or incubation time of the cryopreservation process [9].

51.2.2 The Temperature Changes

Selecting a most suitable cryoprotectant is important, but equally important is the proper selection of the cryopreservation method. The success of cryopreservation depends on the ability of the cryoprotectant to protect spermatozoa from freezing damage, which is reflected in the quality of the sperm after thawing. Based on cooling rates, i.e., the rate by which the temperature is decreased, different sperm freezing methods can be distinguished. The slow-freezing method is based on a slow, gradual decrease in the temperature of the sample, and it can be performed either manually or using an automated system. Sperm vitrification, the non-crystalline, amorphous solidification, is the preservation at extremely low temperatures without freezing. This method is relatively new and the protocol requires further evaluation and standardization for use in spermatozoa. These two methods will be discussed in greater detail below.

51.3 Cryopreservation of Male Gametes

51.3.1 Ejaculated Spermatozoa

The collection of the ejaculated semen after masturbation represents the major part of cryopreservation of male gametes for fertility treatments. The preservation of ejaculated spermatozoa should always be the first option when possible and is indicated whenever a patient can provide a semen sample, which should be collected by masturbation in a sterile plastic container after 2–3 days of sexual abstinence [10]. After a minimum of 20 min of liquefaction at 37 °C, a basic semen analysis should be performed [10]. The semen of the donors must be of good quality according to the WHO guidelines. However, for patients, any sample with adequate spermatozoa motility should be frozen [11]. Since it is possible for men with poor semen quality to have a child by injecting a single sperm into the oocyte (intracytoplasmic sperm injection; ICSI) [12], there is no minimum requirement for semen parameters from patients. Therefore, all samples can be cryopreserved, even those with a low sperm concentration. For men with a disease that affects fertility, the stress of knowing the diagnosis of the disease is itself a physiological and emotional factor contributing to unsuccessful fertilization with an ejaculated specimen, and sometimes, it could even be the reason for the poor quality of the sample. Furthermore, patients who are unable to achieve an ejaculation due to many reasons (social, medical, or religious) and prepubertal adolescents who may be unfamiliar with masturbation can also be considered as candidates for cryopreservation of semen samples. In these cases, it is necessary to use other techniques for sperm collection such as penile vibratory stimulation or electro-ejaculation [13].

51.3.2 Epididymal Spermatozoa

In 1994, Craft and Shrivastav [14] described for the first time percutaneous approaches to retrieve sperm from the epididymis. In cases where a man is diagnosed with azoospermia, it is necessary to use surgical techniques to collect spermatozoa from the testis or the epididymis. In patients with an absence of the vas deferens or an obstruction thereof, epididymal sperm retrieval is indicated. This technique can be performed on the same day when the partner is ovulating or the samples can be frozen prior to the assisted reproduction procedure. Microsurgical epididymal sperm aspiration (MESA) and percutaneous epididymal sperm aspiration (PESA) are the two most commonly used techniques, both of which require local anesthesia.

MESA is a microsurgical technique that requires a small incision in the superior hemiscrotum. The epididymal fluid is aspirated into a tube with sperm medium and subjected to microscopic examination. In contrast, PESA is the direct percutaneous extraction of sperm through the scrotal skin from the epididymis without a microscope. During this procedure, a small-gauge needle is inserted in the caput of the epididymis, and with pressure for several minutes, a clear fluid is aspirated into a syringe. If no sperm are detected in this fluid in a microscopic examination, the needle will be redirected and passed several times through the caput epididymis. With this technique, it is possible to select the most motile sperm from the epididymis. The procedure can be repeated in different parts of the epididymis. If sperm retrieval using MESA fails, either a testicular sperm aspiration (TESA) or testicular sperm extraction (TESE) may be used as alternative procedures.

51.3.3 Testicular Spermatozoa

The retrieval of spermatozoa from men with obstructive azoospermia or non-obstructive azoospermia is restricted to testicular surgery. In cases of obstructive azoospermia at the level of efferent ducts, the sperm can be obtained by TESA or a TESE. Similar to the epididymal retrieval of spermatozoa, TESE can either be performed at the same time as the partner's oocyte aspiration or prior to the oocyte aspiration with subsequent cryopreservation. Previously, TESA was used for diagnostic purposes to evaluate whether or not spermatogenesis takes place in azoospermic patients [15]. To perform a TESA, a needle is inserted in the testicular parenchyma through the scrotal skin, and a negative pressure is applied to aspirate fluid from the seminiferous tubules. Complications related to this procedure are minimal (bleeding (1%) and infection (1%)), and the recovery time is approximately 24 h. In contrary, TESE is a more invasive technique. The procedure for TESE is like that of TESA, but a larger gauge needle is used to obtain multiple biopsies of the testis, and a scalpel is used to make an opening on the scrotal skin. The main risk of this technique is hematoma, and the recovery time is between 24 and 48 h.

51.3.4 Prepubertal Patients and Testicular Tissue Cryopreservation

In 2013, the incidence of cancer in children between the ages of 0 and 14 years was 1% of all the new cancer cases diagnosed in the USA [16]. With safety and efficacy of treatments being established as well as improved survival rates, it is necessary to consider the future quality of life of these children, which includes the preservation of their fertility. However, fertility preservation in children diagnosed with cancer is not yet a standard of care. Besides diagnosis of cancer, some children with non-malignant diseases such as Klinefelter syndrome should be considered for fertility preservation [17].

Since there is no sperm production before the onset of puberty, options for fertility preservation in prepubertal patients are very limited. However, the preservation of

testicular tissue with diploid spermatogonial stem cells has recently been investigated and, to date, has been found to be the only option for this cohort [18]. Immature testicular tissue can be cryopreserved with slow-freezing protocols using dimethyl sulfoxide combined with sucrose or with ethylene glycol as cryoprotectants [17, 19]. Testicular extraction of immature tissue for cryopreservation in boys aged between 3 and 14 years has not shown any acute adverse effects [19]. However, the approaches currently available for gamete maturation are methods that are not well established yet. Therefore, the future fertility of prepubertal patients with frozen immature testicular tissue relies on development of novel methodologies. Although many methods for fertility restoration in young and adolescent male cancer survivors such as auto-transplantation of spermatogonia into the testis, growth and maturation of spermatogonial stem cells in vivo, and auto-transplantation of frozen testicular tissue are already established, these methods need to be further investigated in terms of the fertility potential of these cells. It is also still unclear whether these cells are safe for clinical use.

51.4 Methods for Sperm Cryopreservation

51.4.1 Slow-Freezing

The slow-freezing principle is based on dehydration of cells [20], where slow cooling is combined with low concentrations of a cryoprotectant to achieve an equilibrium. Using this method for cryopreservation, ice crystals are formed at a certain temperature as the temperature is gradually decreasing. The slow cooling is achieved either by a programmable freezer or by manually lowering the samples into liquid nitrogen vapor. Freezing starts with keeping samples at −20 °C for approximately 20 min and then at −86 °C for an additional 60 min. Finally, samples will be stored in liquid nitrogen at −196 °C [6].

Slow-freezing can be performed with fresh, whole ejaculates or washed spermatozoa. It is the preferred method for preservation of human spermatozoa as this method can also be performed with surgically retrieved spermatozoa as well as testicular tissue.

51.4.1.1 Cryoprotectants and the New Generation of Cryopreservation Buffers

Cryoprotectants are used to protect spermatozoa and testicular tissue from damage caused by the ice crystals. The main characteristics of these chemicals are low molecular weight, high cell membrane permeability, and minimal toxicity. The method how the cryoprotectant is added to and removed from the samples is essential to reduce osmotic shock and minimize possible toxic effects. Dimethyl sulfoxide, glycerol, ethylene glycol, and 1,2-propanediol are the more commonly used cryoprotectants. Besides the need for cryoprotection, there is also a need to add cryoadditives. These supplements, also called extenders, have antioxidant effects and are used to improve post-thaw sperm functional parameters such as post-thaw motility, viability, and reducing DNA damage [21].

Glycerol is the most common cryoprotectant used while egg yolk is the most common extender. Since egg yolk contains phospholipids and cholesterol, it increases the tolerance of spermatozoa to cryopreservation [22]. However, one has to consider that extenders from animal origin are a potential source for the transmission of infectious diseases. During the past few years, anti-freeze proteins have been developed with the ability to lower the freezing point, modify the ice crystal formation, avoid recrystallization, and interact with the plasma membrane at low temperatures [23]. However, the use of these proteins for sperm cryopreservation requires further studies and clarification.

51.4.1.2 Slow-Freezing in Vapor Versus Liquid Nitrogen

There are two methods of storage of the cryopreserved samples: immersing in the vapor phase of liquid nitrogen and directly submerging in liquid nitrogen. The option for the storage in the vapor phase seems a more economical option. However, the higher temperature in the vapors may affect the quality of cryopreserved samples. This option can be considered if the samples are kept well within the height of 17 cm from the liquid nitrogen surface and for not more than 3 months [21]. In contrast, storage of the samples directly in liquid nitrogen (−196 °C) is the standard method for long-term storage of semen samples [21]. At this temperature, cellular metabolism and physiological reactions have been slowed down tremendously. Short-term storage of spermatozoa in the vapor phase can replace direct storage in liquid nitrogen to achieve the same results in motility, morphology, and sperm-zona pellucida-binding capacity after thawing [24] (◘ Fig. 51.1). A problem with this method is the possibility of cross-contamination of samples with pathogens from infected samples stored in the same liquid nitrogen container [25]. For this reason, since 1999, the guidelines of the British Andrology Society for the Screening of Semen Donors for Donor Insemination require that cryopreserved gametes have to be stored in sealed vials free from external contamination. Samples that are negative from screened donors have to be stored separately from unscreened individuals [26].

51.4.2 Slow-Freezing Method

Ejaculated semen samples should be collected in a sterile plastic container and liquefied at 37 °C for at least 20 min. After liquefaction, a semen analysis should be performed and freezing must be completed within 1 h. The following protocol (◘ Figs. 51.2, 51.3, 51.4, 51.5, 51.6, 51.7, and 51.8) is for the

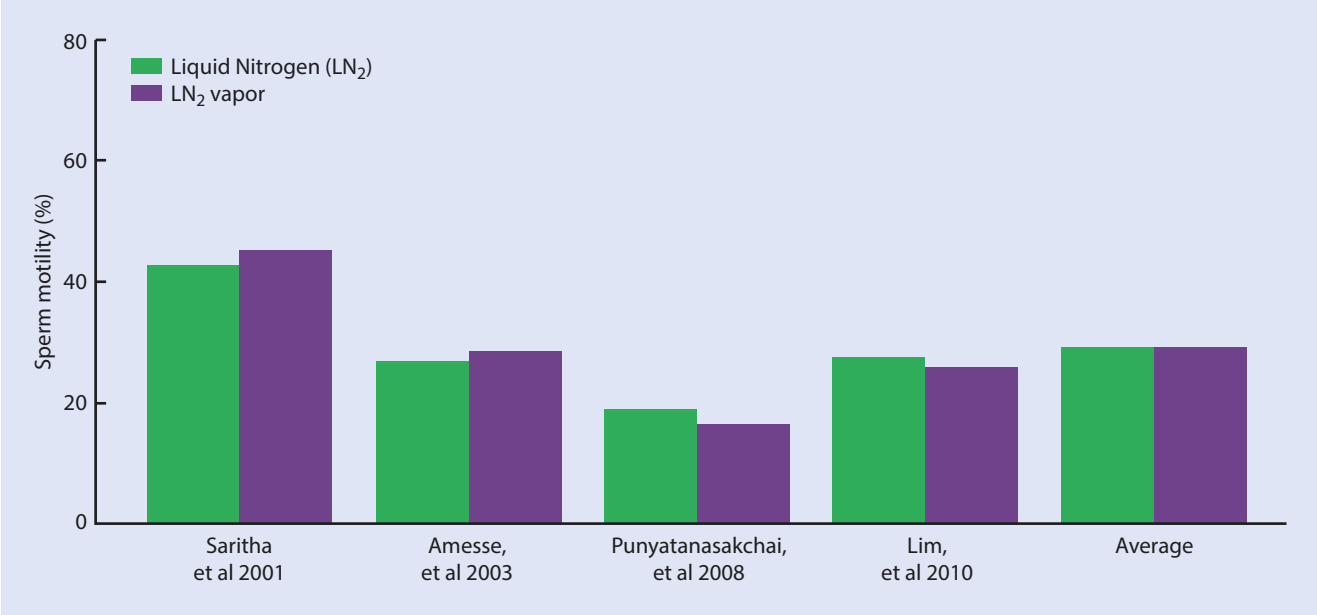

Fig. 51.1 Graphical representation of the effect of sperm preservation in liquid nitrogen compared to liquid nitrogen vapor on sperm motility. The represented studies show no differences between the two options of cryopreservation

Fig. 51.2 A volume of TEST-yolk buffer at 37 °C, equal to 1/4 of the sample volume should be added, then the specimen plus the TEST-yolk buffer is gently rocked for 5 min

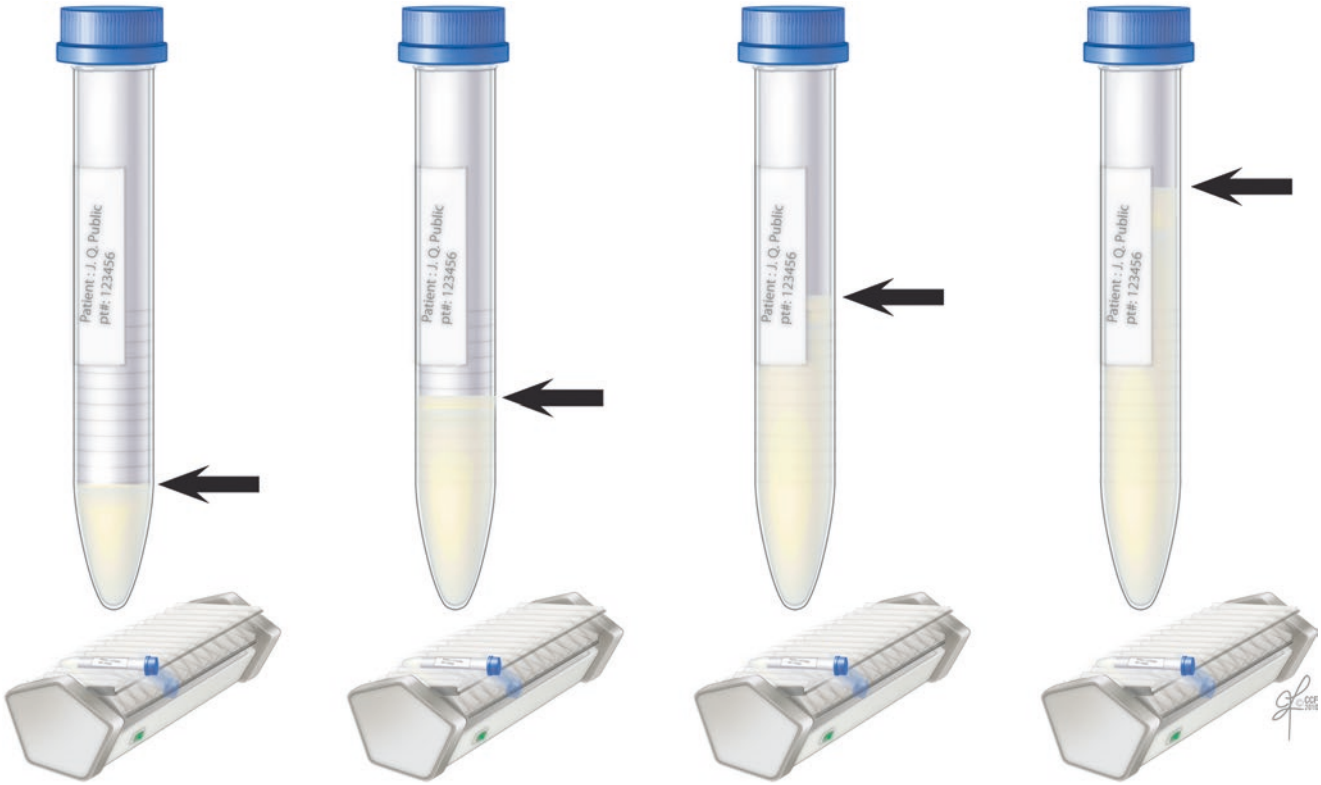

Fig. 51.3 Step-by-step addition of TEST-yolk buffer to the sample. The volume of TEST-yolk buffer should be equal to ¼ volume of the sample

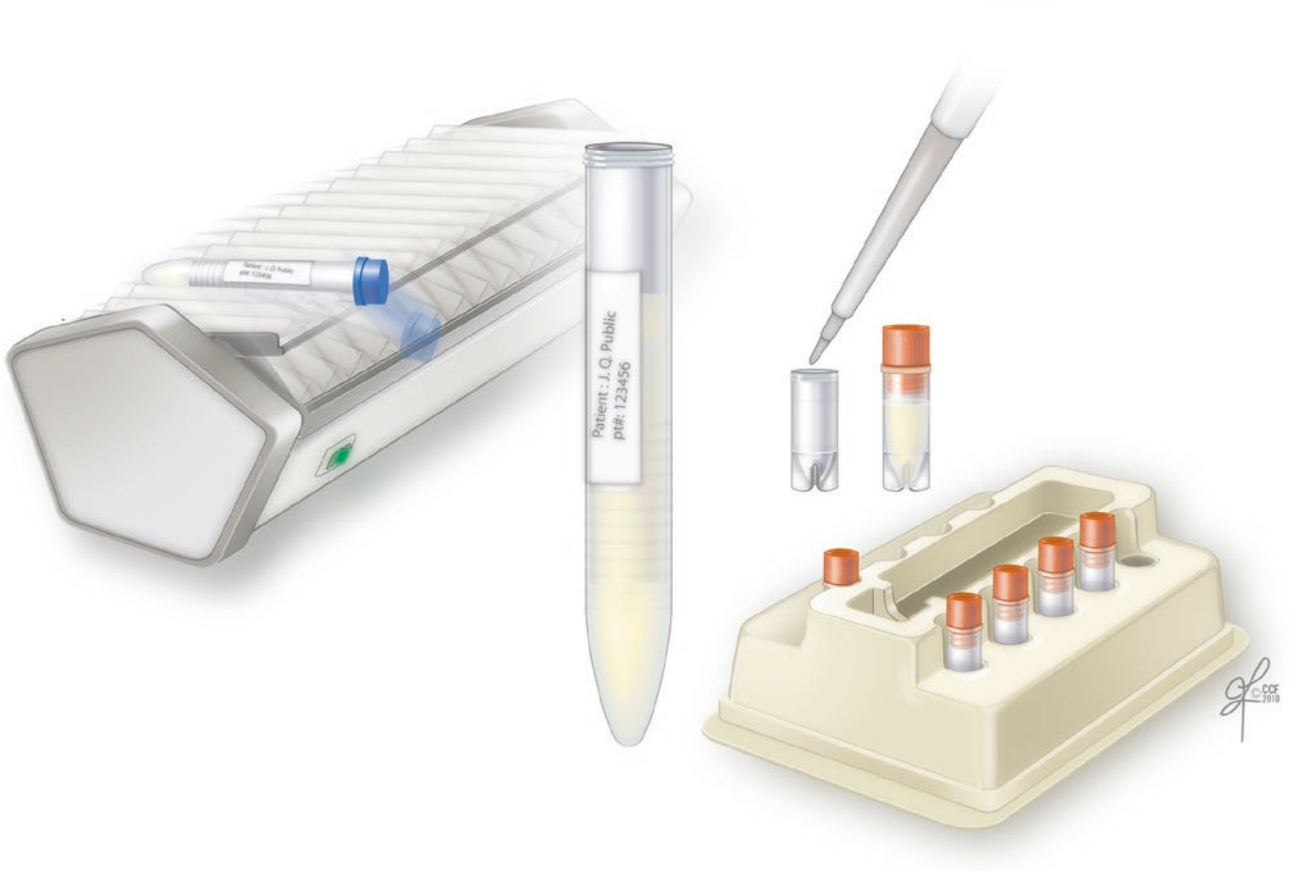

Fig. 51.4 Distribution of the specimen with the freezing medium into cryovials

Fig. 51.5 The cryovials should be placed into the cryocanes before immersion in liquid nitrogen vapors

use of tris, sodium citrate, and fructose (TEST)-yolk buffer as freezing medium supplemented with gentamicin as antibiotic. A semen volume will be diluted with 25% of TEST-yolk buffer at 37 °C and gently rocked for 5 min (Fig. 51.2). This procedure must be repeated three more times until the volume of the freezing medium is equal to the volume of the original semen sample (Fig. 51.3). Subsequently, the samples should be stored in cryovials with a volume not larger than 1.8 ml per cryovial (Fig. 51.4). The tubes should be placed at −20 °C for 8 min (Figs. 51.5 and 51.6) and then kept for at least 2 h in liquid nitrogen vapor (−80 °C) (Fig. 51.7). Finally, the vials have to be transferred into the liquid nitrogen for long-term storage (Fig. 51.8). After 24 h, an aliquot of the frozen sample should be thawed and analyzed [10].

51.4.3 Vitrification

The vitrification process is based on an extremely high cooling rate that prevents ice crystal formation and produces an amorphous, glass-like solid state [27]. Contrary to the slow-freezing method, the effects of the cryoprotectants in spermatozoa are eliminated since this method is cryoprotectant-free. This method of cryopreservation is more commonly used for oocytes and embryos [21], whereas vitrification of spermatozoa is still a challenge, which is attributed to their very specific characteristics. The advantages and disadvantages to slow-freezing method can be found in Table 51.1.

Fig. 51.6 Upright storage during 8 min at −20 °C of the cryovials, with cryocanes and cryosleeves

Fig. 51.7 Vapor phase step in the cryopreservation by slow-freezing process

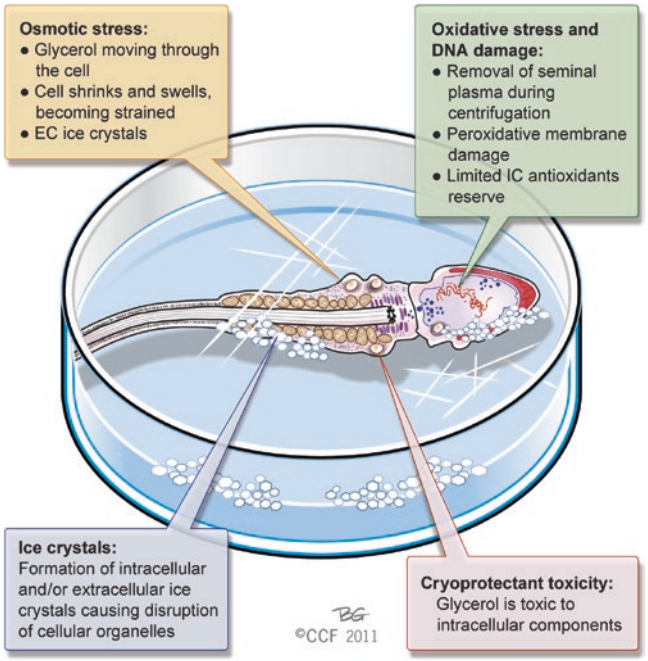

Fig. 51.8 Long-term storage of the samples in liquid nitrogen

Table 51.1 Advantages and disadvantages of slow-freezing vs vitrification

Technique	Advantages	Disadvantages
Slow-freezing	Standardized protocol Fully validated for human spermatozoa storage Approved by the Food and Drug Administration	Expensive and time consuming Use of cryoprotectant Requires a post-thawing process Possibility of cross-contamination
Vitrification	No need for cryoprotectant Simple and quicker Less expensive Free of pathogens Does not require post-thawing process	Inability to preserve larger volumes of sperm Not completely validated for human spermatozoa storage

Since spermatozoa are osmotically sensitive, the use of cryoprotectants can be cytotoxic and cause DNA fragmentation [28]. Isachenko and collaborators reported a cryoprotectant-free vitrification method showing less cryoinjures in human spermatozoa using capillaries [29]. The other limitation of vitrification is the inability to preserve large volumes of sperm. Some studies reported the successful cryopreservation of spermatozoa by vitrification in straws and cryoloop droplets for small volumes [30–32].

For vitrification of sperm, a semen sample can be processed by swim-up and loaded into straws or to a cryoloop container, which ensures that the sample is rapidly cooled by direct contact with liquid nitrogen (−196 °C). After loading the straws or cryoloops, the next step includes the direct contact of the sample with precooled aluminum blocks for a long storage in liquid nitrogen or with a surface of a cryochamber (−180 °C). To the best of our knowledge, no study has reported differences in sperm motility using vitrification compared to slow-freezing (Fig. 51.9).

51.5 Effects of Cryopreservation on Sperm Quality

Intracellular ice formation, osmotic stress, oxidative stress, and cryoprotectant toxicity are some of the factors responsible for the damage in cells during cryopreservation/thawing [33] (Fig. 51.10). Although most of the damage occurs during the thawing process [34, 35], cells can also be damaged due to the formation of intracellular and extracellular ice

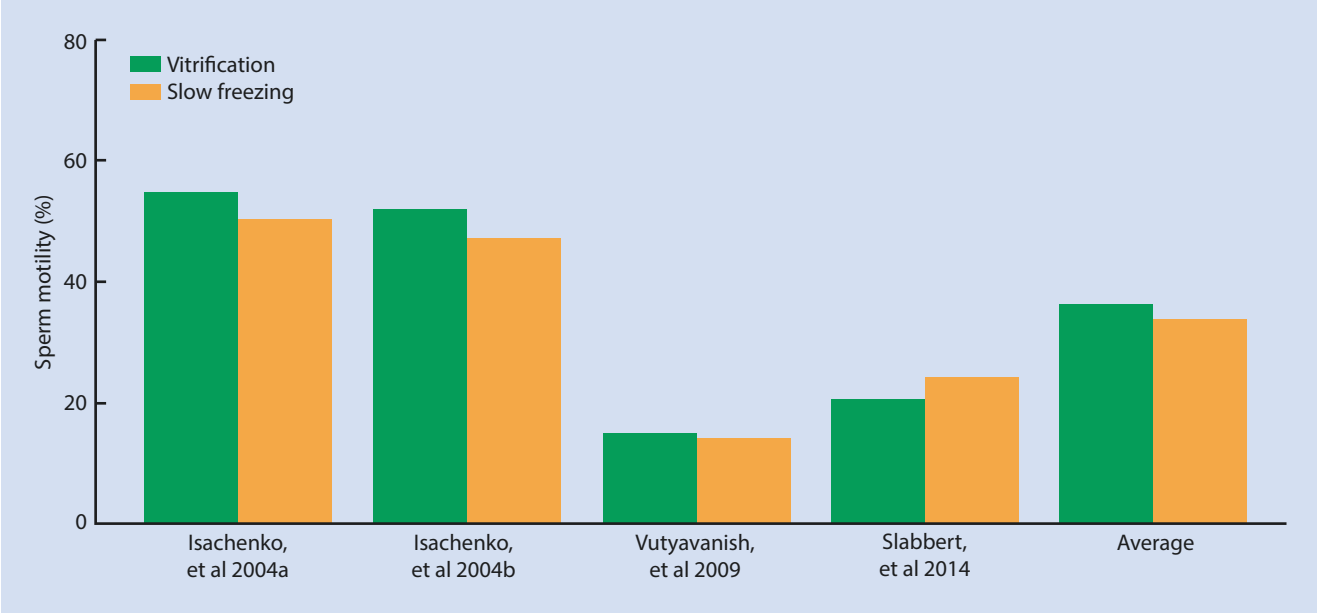

Fig. 51.9 Graphical representation of the effect of sperm cryopreservation using vitrification compared to slow-freezing on sperm motility. The represented studies show no differences between the two options of cryopreservation

Fig. 51.10 Damages in the spermatozoa during cryopreservation

crystals and osmotic stress [36, 37]. These damages cause an imbalance in reactive oxygen species that will affect metabolism and cell signaling, DNA integrity, mitochondrial function, and membrane integrity [31]. The toxicity of the cryoprotectants can be influenced by the slow metabolism of the cells at lower temperatures [38] (Fig. 51.8).

51.5.1 Sperm Parameters

In 1992, MacLaughlin and collaborators [39] reported a decrease in sperm motility after thawing (results from other studies are depicted in Fig. 51.11). Until now, motility is known to be the most affected by cryopreservation/thawing (Table 51.2). A decrease in sperm motility is proposed to be a consequence of mitochondrial damage, sperm tail abnormalities, and changes in the sperm membrane content, such as phospholipids, glycolipids, and sterols [40–42]. It is estimated that sperm cells lose 25–75% of motility after thawing [24, 43–47] (Fig. 51.9). Among the glycolysis taking place in the cellular cytoplasm, mitochondria are also producers of ATP that is essential for sperm motility [21]. Mitochondrial membranes are vulnerable to cryopreservation and consequently the impairment of mitochondria can explain the loss of motility [48]. The fact that the decrease in sperm motility is higher in patients with poor semen quality at the time of sperm banking has to be considered as well [38, 43]. Similar to motility, normal morphology may also decline after thawing [40, 45–47, 49, 50]. Moreover, numerous studies report that the number of viable sperm also significantly decreases after cryopreservation [40, 45–47, 49, 50] (Table 51.2).

51.5.2 Effects on Sperm DNA

The integrity of spermatozoa is an important factor for fertilization. DNA integrity is maintained by the packaging of the DNA in a sperm nucleus. A recent study has attributed the decrease in sperm quality after thawing to sperm DNA fragmentation [21] (Table 51.2), a parameter that, like other basic sperm parameters such as motility, significantly varies from sample to sample [65]. In cryopreserved sperm, DNA integrity can be assessed using a variety of methods. While methods such as terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) directly measure DNA damage [47, 66, 67], the sperm chromatin dispersion

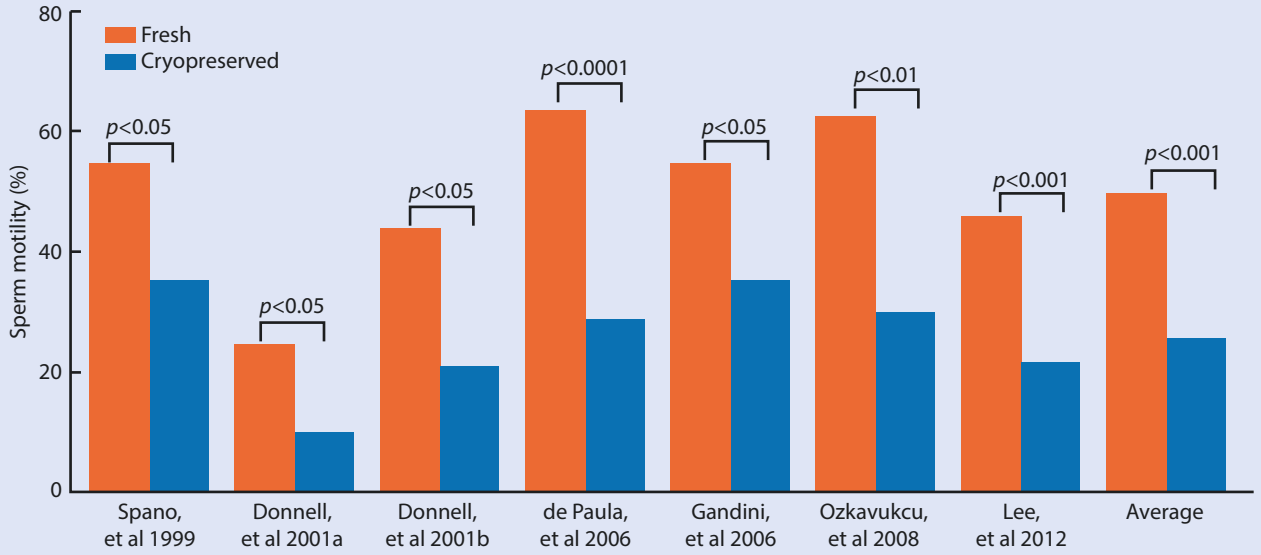

Fig. 51.11 Graphical representation of the effects on sperm motility in fresh spermatozoa and after thawing. A decrease in post-thaw motility was found in all the studies. Significant values found were for $p < 0.05$, $p < 0.01$, and $p < 0.001$

Table 51.2 Reported effects of cryopreservation on sperm parameters

Parameter	Increases	Decline	No change
Volume		[51]	
Concentration		[52]	
Motility		[44], [45], [47], [52], [53], [54], [55], [56], [57]	[58]
Progressive motility		[24], [34], [44], [45], [47], [53], [59], [60], [61]	
Total sperm count		[34], [51]	
Total motile count		[34], [44], [52]	
Morphology		[46], [34], [45], [47], [53], [61]	
Viability		[45], [47], [50], [52], [55], [59], [62]	
DNA fragmentation	[55], [59], [63], [56], [64]		
DNA oxidation	[55]		
Sperm chromatin packaging		[46], [50], [47],	
Membrane integrity		[46]	[57]
DNA integrity		[34][b], [60]	[34][a]

[a]Fertile men
[b]Infertile men

test (SCD) or the COMET assay measure the damage after denaturation [67, 68]. There are also other methods available, which are based on emission spectra of dyes sensitive to the status of DNA-associated protamines, such as the acridine orange assay [67, 68]. Since the oxidant levels are increased in sperm after cryopreservation [69], this results in oxidative stress, which is mainly responsible for increased DNA fragmentation [34, 55] in cryopreserved sperm [55, 70]. Sperm from infertile men or men with poor semen quality have more DNA damage after cryopreservation [11, 55, 70], while samples from infertile men per se have an increased state of oxidative stress [71–73].

51.5.3 Effects on Pregnancy Outcome

Many studies have reported pregnancy outcomes after using fresh or frozen ejaculated spermatozoa [21, 38, 74] (Table 51.3) (Figs. 51.12, 51.13, 51.14 and 51.15). A study in patients with non-obstructive azoospermia using frozen-thawed spermatozoa showed higher implantation and pregnancy rates when compared to fresh spermatozoa [75]. In contrary, a more recent study in 2016 found no differences in ART rates after ICSI between fresh or cryopreserved spermatozoa from patients with Klinefelter syndrome [76]. Meta-analyses comparing the use of fresh vs frozen spermatozoa in ICSI reported no statistical differences in the assessment of clinical fertilization and pregnancy rates between the two groups [74, 77].

ART performed with cryopreserved spermatozoa obtained before gonadotoxic treatment in patients with cancer is reportedly successful. However, the success rate in patients with cancer related to lymphoma or the testis is slightly lower [52, 78, 79]. Some studies report lower or no statistical differences in the fertilization rates with cryopreserved testicular/epididymal spermatozoa from azoospermic men, when compared with freshly obtained germ cells [74, 80]. Besides the use of frozen or fresh spermatozoa, the quality of spermatozoa at the time of usage and the ART technique used are crucial to achieve a pregnancy with success [38, 81–83] (Figs. 51.12 and 51.13).

Table 51.3 Reported effects of cryopreservation in fertilization and pregnancy rates

Parameter	Increases	Decreases	No changes
Embryo cleavage			[80], [84], [85], [86], [87], [88]
Fertilization rate		[84][a], [85]	[75], [80], [84][b], [86], [87], [89]
Implantation rates	[75]		[84], [87], [90], [88], [91]
Pregnancy rates	[75], [89][c], [92][a]	[89]	[80], [84][a], [86], [92][a], [93], [85], [94], [90], [88], [91]
Take-home babies			[75], [80], [91]

[a]Epididymal sperm
[b]Testicular sperm
[c]Non-obstructive azoospermia

51.5.4 Length of Freezing and Effects on Sperm Quality

The most common reason for long-term storage of sperm is cancer. Semen and spermatozoa from these men can be cryopreserved before cancer treatment [18, 38, 95]. A study conducted in 2013 reported the birth of twins after in vitro fertilization using semen stored 40 years ago [96]. Some long-term follow-up studies are reporting on sperm quality after thawing of cryopreserved semen from oncological patients [52, 79, 97–99]. Meseguer and collaborators reported that sperm characteristics after approximately 3 years of storage show no differences after thawing in total

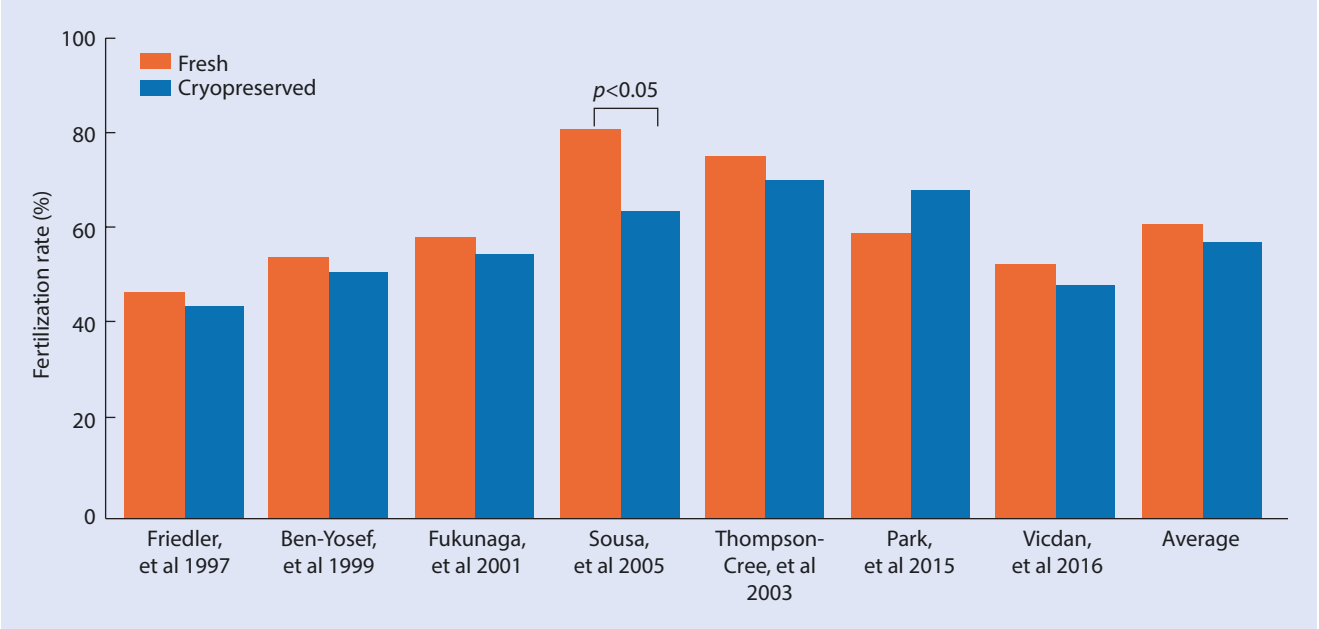

Fig. 51.12 Graphical representation of the fertilization rate in ART using fresh spermatozoa in comparison to cryopreserved spermatozoa. Only one study (Sousa, et al. 2005) reported a higher fertilization rate using fresh spermatozoa, with a $p < 0.05$

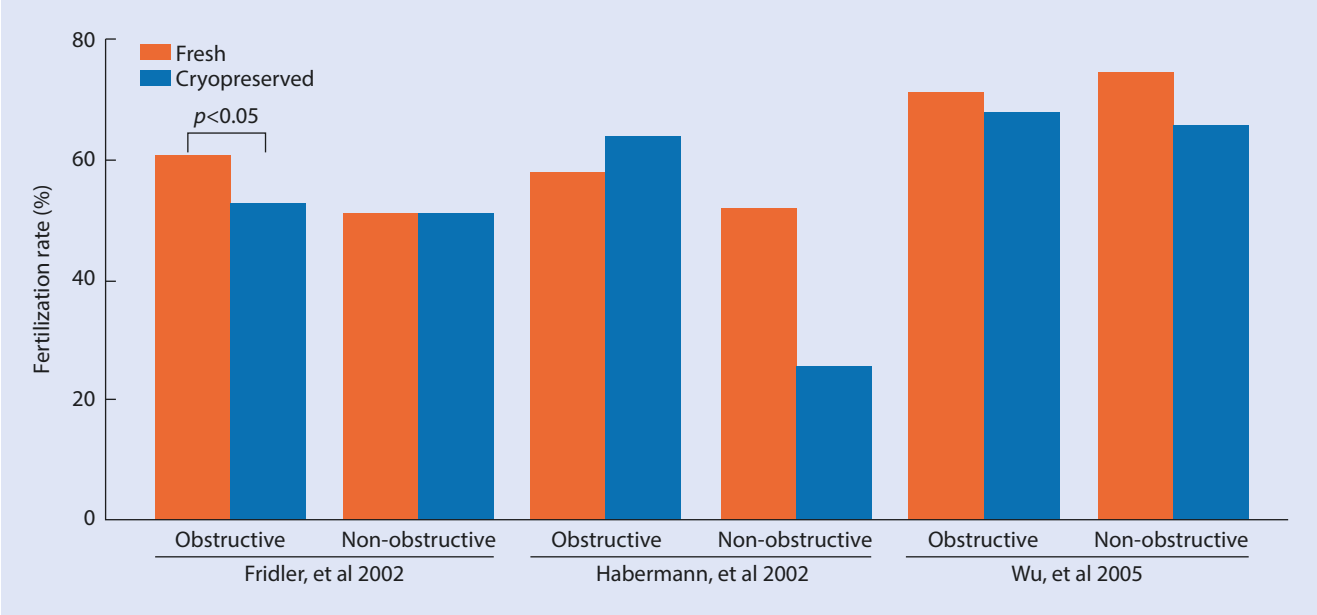

Fig. 51.13 Graphical representation of ART fertilization rates using obstructive and non-obstructive azoospermia and comparing the use of fresh and cryopreserved spermatozoa in each case. In case of obstructive azoospermia, only one study (Fridler et al. 2002) reported a higher fertilization rate using fresh spermatozoa, with a $p < 0.05$

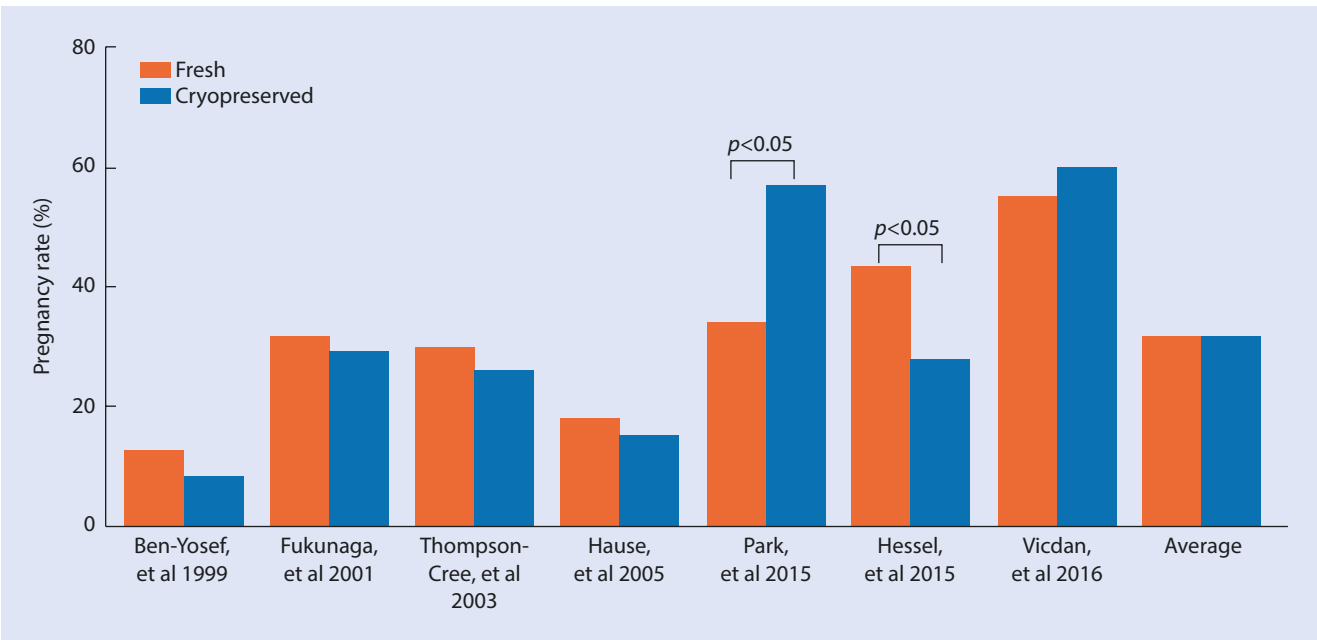

Fig. 51.14 Graphical representation of pregnancy rates in ART comparing the use of fresh vs. cryopreserved spermatozoa. Two studies show contrary results: Park et al. (2015) showed an increase in pregnancy rates using cryopreserved spermatozoa, while Hessel et al. (2015) reported a decrease in pregnancy rates, with a $p < 0.05$

sperm count and motility between cancer patients and controls [51]. Yogev and coworkers reported no differences in motility between different lengths of cryopreservation [100]. Another study also reports no differences between sperm parameters from men with testicular cancer, Hodgkin's disease, and other cancers after approximately 10 years of cryopreservation [101].

51.6 Sperm Cryopreservation

51.6.1 Sperm Donors

Currently, the use of donor sperm in assisted reproduction programs is a common practice [5, 6, 26, 102, 103]. It is mandatory to screen the donors for any infection or

genetic disorders. For a semen sample to be accepted, the concentration of progressively motile sperm after thawing is the most important parameter to be considered [104]. Among the examples of couples who request sperm donor samples are partners of patients with azoospermia, genetic diseases, HIV-positivity, and same-sex couples. In the USA, some agencies provide extensive non-identifying information [105, 106]. These agencies will counsel recipients of sperm donations about the cultural and psychological background of the donors, and when the child reaches the age of 18, the agencies will provide the name and other details of the donor [103, 105, 106]. This will allow the offspring to find the father, if they should so desire [103, 106].

51.6.2 Cancer Patients

Cancer is the major reason why men cryopreserve their gametes [107, 108], and an estimate of 900,000 men in the USA were expected to be diagnosed with cancer in 2018 [109]. Despite the fact that exposure to chemo- and radiotherapy is responsible for the loss of fertility in men undergoing cancer treatment [107], only 48% of the physicians discuss the possibility of sperm banking with the patients and only 25% with cancer patients [108]. This loss of fertility may be permanent or temporary [110, 111]. Therefore, sperm banking before cancer treatment is always recommended, even when the sperm parameters are poor, as it is in the case of reproductive cancers [107, 108]. When cancer is diagnosed in pediatric patients, where the adolescents have not achieved puberty, cryopreservation of testicular tissue is an option [112–115], which should always be discussed with the parents and the child [38, 116].

51.6.3 Other Diseases

There are several non-reproductive diseases, such as Klinefelter syndrome [76], which can be responsible for the impairment of fertility, in which case sperm banking is recommended as soon as possible. Infertility can also be caused by the treatment of a condition, such as autoimmune disorders [117], diabetes, or heart transplant where gonadotoxic drugs [118, 119] are used. In these cases, sperm banking is recommended prior to therapy [120, 121].

51.6.4 Traveling Partner

Sperm cryopreservation in case of a traveling partner allows the utilization of male gametes at the time of female ovulation. This can be an option when the partner is absent at the time of ovulation, due to deployment in the military, or due to frequent or long business trips [121].

51.6.5 Pre-vasectomy

Vasectomy is a male contraceptive method where the *vas deferens* is cut and the ends closed or cauterized (blocked) resulting in the absence of spermatozoa in the ejaculate [122]. Many men who go for vasectomy want to perform sperm banking prior to vasectomy [123]. This option allows them to have their biological children later on, should their life circumstances or demographics change [38].

51.6.6 Transgender

Male-to-female transsexualism (transwomen) results in an irreversible sterile condition. Furthermore, transwomen often wish to become biological parents and start a family, like other people at the reproductive age. It is only in the last 10 years that these individuals are also eligible for this option to preserve their fertility [124]. It is also known that parenting is one of the preventing factors for suicide in transwomen [125]. Now, transwomen can preserve their fertility using the sperm banking option before undergoing any medical transition procedures [126].

51.7 Postmortem Utilization of Cryopreserved Semen

Viable sperm can be retrieved from a cadaver up to 24–36 h postmortem and can still be frozen [127, 128]. The use of posthumous sperm raises many controversial, ethical, and legal concerns about the ownership of the sperm [108]. High fertilization rates (40–100%) using postmortem sperm have been reported [129]. In cases of patients who cryopreserved spermatozoa prior to a medical treatment, in case of death, clear and precise instructions about the use of the sample and an informed written consent that is recognized by law have to be obtained from these patients [130].

51.8 Future Developments of Cryopreservation in Male Patients

When using conventional sperm cryopreservation protocols, a decline in viability, chromatin integrity, motility, and mitochondrial potential is observed. It is accepted that most of the damages occur during the thawing process. Optimization of the freezing medium with antioxidant supplements is a promising option. Newer freezing techniques such as vitrification also appear to be promising as they are less time-consuming and require minimal processing of the sperm. Still, the size of the sample and cross contamination are major concerns with the use of open devices for this technique. Therefore, further studies are necessary to assess the fertilization and pregnancy rates using vitrified sperm. Another technique with potential, though still in the development phase for

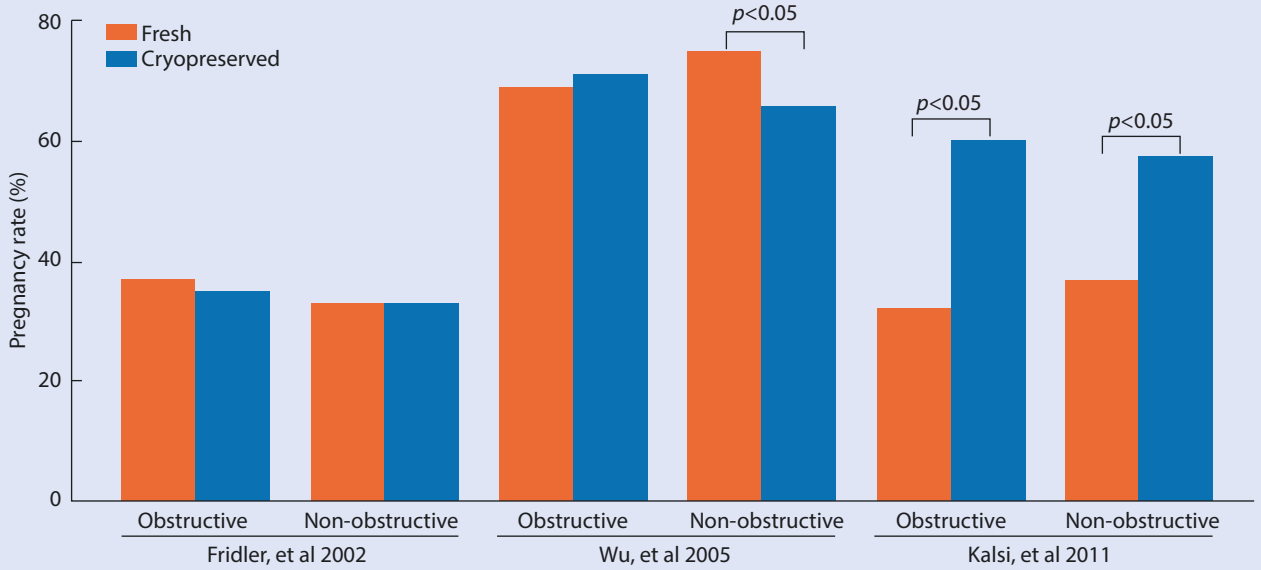

Fig. 51.15 Graphical representation of the pregnancy rates in ART using obstructive and non-obstructive azoospermia and comparing the use of fresh and cryopreserved spermatozoa in each case. In case of obstructive azoospermia, only one study (Kalsi et al. 2011) reported a higher pregnancy rate using fresh spermatozoa, with a $p < 0.05$. In case of non-obstructive azoospermia, Wu et al. (2005) demonstrated a decrease in pregnancy rates using post-thaw spermatozoa, while Kalsi et al. (2011) found increased pregnancy rates using post-thaw spermatozoa, with a $p < 0.05$

human spermatozoa, is lyophilization (freeze-drying) of sperm where the samples can be stored at 4 °C [21, 131]. After lyophilization, the spermatozoa are immotile, but the DNA damage is less when compared with liquid nitrogen freezing [132]. However, since the spermatozoa are immotile, lyophilized sperm can only be used in ICSI.

51.9 Conclusion

Cryopreservation of male spermatozoa is the standard of care for fertility preservation in patients undergoing to gonadotoxic treatments, such as chemotherapy/radiotherapy. There are other scenarios where a couple or an individual patient will need to cryopreserve the spermatozoa, for example, before vasectomy or in cases of a traveling husband. With the development of medicine and research, newer opportunities for cryopreservation have been explored. The option for cryopreservation of testicular tissue in cases of pediatric cancer provides hope for patients of fathering a child in the future. With modern societies being more open and liberal, the wrong sexual identity of individuals is considered a problem, and these individuals do try to have their physical gender changed. Consequently, transwomen should have an opportunity to cryopreserve spermatozoa before undergoing gender reassignment.

The conventional cryopreservation (slow-freezing) protocol is standardized and widely used. However, challenges remain in terms of functional sperm parameters including motility after the thawing. Although new methods to preserve spermatozoa are promising, they still need validation before being routinely used for cryopreservation of spermatozoa in an assisted reproduction program. These new methods include the necessity for the development of new cryoprotectants and new antioxidants to improve sperm quality after thawing. Vitrification is a new technique of cryopreservation, which is being investigated in recent years. While this method is commonly used for oocytes and embryos, for spermatozoa, challenges remain due to the special characteristics of male germ cells. This technique is further limited as only small volumes with small numbers of spermatozoa can be cryopreserved. Lyophilization of spermatozoa is another method that is still being investigated. The problem associated with this technique is the fact that male germ cells are completely immotile after lyophilization and can therefore only be used for ICSI. Hence, future research needs to be conducted not only on the optimization and safety but also on the health of the offspring.

Review Questions

1. Which of the following characterizes a good cryoprotectant?
   (a) Low toxicity
   (b) High toxicity
   (c) High pH
   (d) None of the above

2. Short-term storage of cryopreserved samples in vapors can be considered as an option with the following conditions:
   (a) Height of 3 cm from the liquid nitrogen surface and for not more than 17 months
   (b) Height of 7 cm from the liquid nitrogen surface and for not more than 3 months
   (c) Height of 27 cm from the liquid nitrogen surface and for not more than 7 months
   (d) Height of 17 cm from the liquid nitrogen surface and for not more than 3 months

3. The estimated percentage loss of motility after thawing is between:
   (a) 15 and 65%
   (b) 5 and 20%
   (c) 25 and 75%
   (d) 80 and 100%

4. It is mandatory to freeze the sperm from a donor in order to:
   (a) Allow time to screen for any infection
   (b) Allow time to screen for genetic disorders
   (c) Allow time to analyze the physical characteristics of the donor
   (d) a and b are correct

5. To avoid concerns about the legal ownership of the cryopreserved sample in case of use of posthumous sperm:
   (a) An informed written consent recognized by law should be provided to the patient.
   (b) An informed written consent should be provided to the patient.
   (c) An informed oral consent recognized by law should be provided to the patient.
   (d) An informed oral consent recognized should be provided to the patient.

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Technologies for Cryoprotectant-Free Vitrification of Human Spermatozoa: Asepticity as a Criterion for Effectiveness

Vladimir Isachenko, Gohar Rahimi, Peter Mallmann, Raul Sanchez, and Evgenia Isachenko

Authors Vladimir Isachenko, Gohar Rahimi, Peter Mallmann, R. Sanchez, and Evegenia Isachenko are contributed equally to this work.

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Learning Objectives

In natural conditions, the egg and embryo are subjected to ever-changing dynamic processes. However, the routine assisted reproductive technologies (ARTs) used today involve the use of static in vitro culture systems. The objective of this work is to determine whether there is any difference in the viability of embryos after in vitro culture under static and mechanical microvibration conditions.

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52.1 One Hundred and Twenty Years of Cryoprotectant-Free Vitrification of Human Spermatozoa

As noted by Parkes [1], Davenport reported in 1897 that human spermatozoa would survive freezing down to −17 °C. However, these extremely interesting data were forgotten for 40 years.

In 1938, Jahnel cryopreserved human spermatozoa at −79 °C, at –196 °C (with 52 h of storage in liquid nitrogen), and at −269 °C (with 3 h of storage in liquid helium). For cooling containers he used vials of Duran glass (today this polymeric material, invented shortly before 1938, is known as plexiglass). In these tubes, native spermatozoa were directly plunged into the cooling agent. After warming the samples at 37 °C for some minutes, the author noted that some spermatozoa displayed high activity similar to that in fresh ejaculate.

In 1940, Shettles repeated the experiments of Jahnel [2], but cooling of the sperm was performed in capillaries 0.2 mm in diameter. A few of the spermatozoa that were revived after warming were viable (cited by Parkes [1]).

In 1942, Hoagland and Pincus described freezing of human and rabbit spermatozoa, using a bacteriological loop to cool small specimens in rabbit serum with rapid cooling. They obtained up to 40% viable human spermatozoa after cooling a sperm film in liquid nitrogen followed by rapid warming of these microvolumes [3].

The subsequent decrease in interest in cryoprotectant-free vitrification of human spermatozoa can probably be explained by the beginning of the era of permeating cryoprotectant use in cryobiology.

In 1937, Bernstein and Petropavlovski reported that spermatozoa of many mammalian species (bulls, rams, stallions, boars, and rabbits) were cryopreserved successfully with cooling down to −22 °C, using 0.5–2.0 M glycerol.

In 1949, Polge et al. [4] used glycerol for deep freezing of fowl spermatozoa. From that time until now, in the management of human male infertility, cryopreservation of spermatozoa by conventional freezing with permeating cryoprotectants has become a routine and effective technique.

Sixty years after the publication of the report by Hoagland and Pincus [3], the results of investigations aimed at comparing the effectiveness of three methods of cryopreservation of human spermatozoa—conventional freezing with permeating cryoprotectants, cryoprotectant-free vitrification, and vitrification with permeating cryoprotectants—were published [5].

In these experiments [5], ejaculate samples containing at least 20 million spermatozoa per milliliter, with at least 50% progressive motility and 15–30% morphologically normal spermatozoa after swim-up, were frozen "slowly" and vitrified.

For "slow" (programmable or conventional) freezing the samples in both groups (native and swim-up-prepared spermatozoa) were divided into four subgroups: native spermatozoa without cryoprotectant, native spermatozoa with cryoprotectant, spermatozoa after swim-up without cryoprotectant, and spermatozoa after swim-up with cryoprotectant. Test-egg-yolk buffered-glycerol freezing medium (TYBG; Scandinavian IVF Science, Gothenburg, Sweden) was used. After 1:1 dilution with TYBG, 0.25 mL of the sperm suspensions was pipetted into standard 0.25-mL insemination straws and kept at room temperature for 10 min. Then the straws were placed in a programmable freezer and cooled at −10 °C/min. Thawing of the samples was performed by plunging the straws into a water bath at 37 °C for 50 s.

For cryopreservation by direct plunging into liquid nitrogen (vitrification), samples from both groups were divided into four subgroups: native spermatozoa without cryoprotectant, native spermatozoa with cryoprotectant, spermatozoa after swim-up without cryoprotectant, and spermatozoa after swim-up with cryoprotectant. TYBG medium, in the same concentration as that used for slow freezing, was used for vitrification with cryoprotectant. Samples of the spermatozoa were placed onto copper loops 5 mm in diameter (drops 20 μL in volume) or into standard 0.25-mL insemination straws. The loops and straws were plunged into liquid nitrogen.

Thawing of the loops was performed by plunging them into a 15-mL tube with 10 mL of strongly agitated culture medium at 37 °C. After thawing of five loops in one tube, this tube was put into a CO_2 incubator at 37 °C for 5–10 min. Then the spermatozoa were concentrated by centrifugation at 380 × *g* for 10 min. The pellet was resuspended in 100 μL of culture medium.

Thawing of the straws was performed by plunging them into a water bath at 37 °C for 50 s. The test for evaluation of dead and acrosome-reacted spermatozoa was performed according to the method described by Talbot and Chacon [6], with staining in 2% trypan blue, fixation in 3% glutaraldehyde, and staining with Bismarck brown. The colors of the acrosomal and postacrosomal regions of the spermatozoa were evaluated. Dead spermatozoa have a dark blue to black postacrosomal region and a pink or white acrosomal region. Live spermatozoa without an acrosome reaction at the time of fixation have a light brown postacrosomal region and a pink acrosomal region. Live spermatozoa with a normal acrosome reaction at the time of fixation have a light brown postacrosomal region and a white acrosomal region. Sperm morphology was assessed according to strict criteria [7].

Also, the ability of fresh (control) spermatozoa and spermatozoa vitrified without cryoprotectant to fertilize oocytes was evaluated.

It was established that the motility rates of the spermatozoa were 55% in the fresh native sperm samples and 87% after swim-up. Ten minutes after dilution with TYBG, the rates of motility were nonsignificantly reduced to 42% in native spermatozoa and significantly reduced to 73% in swim-up-prepared spermatozoa. These data demonstrated that addition and removal of cryoprotectant can negatively influence the motility of spermatozoa. Programmable freezing without cryoprotectant reduced the rates of sperm motility significantly to 1% in native spermatozoa and 0.2% in swim-up-prepared spermatozoa. After this type of cryopreservation using cryoprotectant, in comparison with nonfrozen samples, the rates of motility were reduced to 16% in native spermatozoa and 36% in swim-up-prepared spermatozoa. The proportions of spermatozoa with normal morphology were decreased to 12% in native spermatozoa and 12% in swim-up-prepared spermatozoa.

After vitrification in standard 0.25-mL straws, nearly all spermatozoa (98%) were immotile. The highest motility rate after vitrification was achieved with swim-up-prepared spermatozoa on copper loops without cryoprotectant (50%). This rate was lower (38%) after conventional freezing of swim-up-prepared spermatozoa with cryoprotectant. For conventional freezing with cryoprotectant, the highest post-thaw rates of motile spermatozoa recovery were obtained with native spermatozoa (45%) and swim-up-prepared spermatozoa (55%).

After vitrification on copper loops, the highest post-thaw rate of motile spermatozoa recovery was achieved with swim-up-prepared spermatozoa without TYBG treatment (57%). The highest proportion of morphologically normal spermatozoa was obtained after conventional freezing of TYBG-treated/swim-up-prepared samples (27%). After vitrification of swim-up-prepared spermatozoa without cryoprotectant, this parameter was not significantly different (26%).

The proportions of "viable" cells were comparable (59%) after programmable freezing and after vitrification of swim-up-prepared spermatozoa without cryoprotectant. The highest proportion of acrosome-reacted cells was found in swim-up-prepared samples after vitrification without cryoprotectant (38%). It was shown that preservation of the fertilization properties of spermatozoa after vitrification resulted in a higher rate of embryo formation than that observed in native (control) spermatozoa.

It was concluded than cryoprotectant-free vitrification of human spermatozoa is a perspective direction of cryopreservation [5].

52.2 Conventional Freezing and Vitrification with Permeating Cryoprotectants Versus Cryoprotectant-Free Vitrification

Conventional freezing with permeating cryoprotectants leads to mechanical cell injury, which is a consequence of intracellular and extracellular ice crystal formation and osmotic damage due to extensive cell shrinkage. Subsequent rewarming and thawing of the cells can cause further deterioration in their viability through possible excessive osmotic swelling [8–10]. As a result, the average velocity in terms of the percentage of motile spermatozoa drops significantly after cryopreservation relative to that of fresh sperm (Watson et al. 1995; [11–14]).

It has been established that the production of reactive oxygen species leads to an increase in lipid peroxidation after cryopreservation of spermatozoa [15] and that this event is associated with a loss of sperm motility [16, 17]. This type of cryopreservation with permeating cryoprotectants can provoke extensive chemical and physical damage in spermatozoa cell membranes as a result of changes in lipid-phase transition and increased lipid peroxidation.

Alvarez and Storey [16, 18] and Chatterjee and Gagnon [19] have suggested that the injury to human spermatozoa induced by conventional freezing occurs mainly during thawing, and it has been related to diminished antioxidant defense activity during cooling and/or structural damage to the cytoskeleton and/or antioxidant enzymes during cryopreservation [16, 18].

This suggests that conventional ("slow") freezing and thawing of spermatozoa, aside from ice crystal formation, is intrinsically deleterious. To prevent excessive cell shrinkage during conventional cryopreservation, permeating cryoprotectants are used. However, the effectiveness (prevention of intracellular ice formation) of permeating cryoprotectants during this type of freezing can be achieved only with a low cooling rate [9], which, as mentioned earlier, can be damaging in itself. Besides, the introduction (before cooling) and removal (after thawing) of permeating cryoprotectants can produce damage even at room temperature in the absence of freezing or thawing (or both). The main mechanisms of permeating cryoprotectant toxicity have been discussed elsewhere [20] and include osmotic damage, as well as chemical toxicity to cells and membranes [19, 21, 22].

Conventional freezing and thawing, with all of these negative effects, can also lead to chromatin damage. In view of this possibility, assessment of sperm nucleus integrity is very important because chromatin abnormalities have repercussions for sperm quality and male fertility status [23].

Besides, sperm DNA damage is strongly correlated with mutagenic effects of conventional freezing [24]. In fact, freezing and thawing of spermatozoa from fertile and infertile men lead to chromatin damage, as well as effects on spermatozoa morphology and membrane integrity [25–28].

It has been demonstrated that any defects in the chromatin structure of spermatozoa from infertile men showing increased DNA instability are sensitive to denaturing stress [29]. This stress may be induced by several treatments, including conventional freezing. Despite this, oocytes have the ability to repair a small amount of sperm DNA damage, although this seems to be insufficient to support subsequent embryo development [30], and DNA damage can lead to decreased conception rates or conception failure [28, 31]. The percentage of spermatozoa with fragmented DNA has also been negatively correlated with the success of

in vitro fertilization and intracytoplasmic sperm injection (ICSI) [32].

In contrast to conventional freezing ("slow" ice-forming techniques), the protocols for vitrification currently used for cryopreservation of oocytes and embryos, as a rule, involve the use of very high concentrations (25–50%) of permeating cryoprotectants and relatively high cooling rates in comparison with the rates used in conventional freezing.

At the same time, the presence of permeating cryoprotectants substantially decreases the critical rates of freezing and warming. However, it is known that high concentrations of cryoprotectants have a toxic effect [33–36].

52.3 Cryoprotectant-Free Vitrification of Human Spermatozoa Can Entail the Use of Nonpermeating Compounds: Carbohydrates, Proteins, Lipoproteins, and Antioxidants

It is known that for cryopreservation of mammalian and human spermatozoa, mixtures of nonpermeating compounds—such as proteins (e.g., in the form of human serum albumin (HSA)) for antioxidant and cryoprotectant purposes, and sucrose as the agent for dehydration of cells—can be used [37–51].

During vitrification with permeating cryoprotectants, carbohydrates play a role in compensating for the change in osmotic pressure caused by the permeating cryoprotectants, increasing this pressure during saturation with the cryoprotectants (dehydration) and decreasing during removal of the cryoprotectants (rehydration).

What roles do carbohydrates play in cryoprotectant-free vitrification when we use only isotonic solutions?

The literature answers on this question are a little unclear. Carbohydrates can stabilize the cellular membrane. For example, there is an opinion that raffinose plays the role of a stabilizer of the mouse oocyte membrane [52, 53]. Koshimoto and Mazur [52] investigated three different sugars (monosaccharide glucose, disaccharide sucrose, and trisaccharide raffinose). It was shown that protection against cryodamage during cryopreservation (freezing and thawing) depended not on the type of sugar or its molar concentration, but on the mass concentration of sugar. The positive effect of sucrose on the cell plasma membrane has been described by Rodgers and Glaser [54].

Lipoproteins in seminal plasma maintain the lipid composition of the plasma membrane and increase the viability and cryostability of the cellular membranes of spermatozoa during storage and cryopreservation [55]. Probably the same positive roles are played by lipoproteins for stabilization of mitochondrial membranes.

Antioxidant properties of seminal plasma have been described in humans [51], rats [38], and mice [41]. According to Sasaki et al. [46], the antioxidant properties of seminal plasma are attributable to low molecular weight protein fractions, whereas Liu et al. [44] suggest that antioxidant protection of spermatozoa is related to the proteins in seminal plasma.

52.4 Extremely High Viability of Viruses, Bacteria, Mycoplasmas, and Fungi After Cryoprotectant-Free Cryopreservation

What happens to microorganisms when they get into liquid nitrogen spontaneously (cryoprotectant-free vitrification) in culture medium or even in distilled water?

In fact, *viruses* (especially enveloped ones) present big problems in medicine.

Speck et al. [56] showed that certain cryoprotectant-free suspension media (skim milk, buffered saline, gelatin, rabbit serum, egg yolk, allantoic fluid) permit adequate storage of *herpes simplex virus* in dry ice (−70 °C) for many months.

Melnik [57] studied enveloped viruses (*herpes*, *measles*, *sindbis*, and *vesicular stomatitis viruses*) as well as nonenveloped viruses (*vaccinia virus*, *adenovirus*, and *poliovirus*). For cryoprotectant-free freezing, the viruses were mixed with distilled water and 20% fetal calf serum. Then samples were frozen by reduction of the temperature from 25 °C to −40 °C over an interval of 20 min. The viruses were thawed rapidly at 37 °C. It was established that enveloped viruses were fully inactivated after freezing in distilled water; however, after freezing in 20% fetal calf serum, these viruses were only partially inactivated after freezing and thawing. Even after four cycles of freezing–thawing, positive titer values were noted. Thus, it was concluded that fetal calf serum can protect enveloped viruses (during freezing in the absence of permeating cryoprotectants).

The nonenveloped viruses were not inactivated by freezing and thawing, even in the absence of protective agents (in distilled water). After cryoprotectant-free freezing in 20% fetal calf serum, two of the three tested viruses (*vaccinia virus* and *poliovirus*) were activated. The stable characteristics of these viruses are well known [58].

In our opinion, the following technology, which is intensively used in the biomedical industry, can demonstrate extremely high cryostability (cryoresistance) of viruses. This technology is freeze-drying (lyophilization) of live virus vaccines [59] and entails (at the beginning of vacuumization) the freezing of enveloped viruses in a cryoprotectant-free solution. These viruses have lipid membranes and a water volume confined by these membranes (in cells, this volume is the cytoplasm). For protection of these viruses, a number of nonpermeating compounds are used: mannitol, sorbitol, sucrose, gelatin, glutamate, HSA, and fetal bovine serum.

We would like to underline here that the most "famous" viruses (*human immunodeficiency virus* (HIV) and *hepatitis virus*) are enveloped viruses. For nonenveloped viruses, the question of cryopreservation a priori is absent because they are lipid membrane free and water free. For these viruses, "cryopreservation" means only cooling (e.g., by direct plunging into liquid nitrogen) and nothing more.

Experiments by Stringfellow et al. [60] demonstrated the high level of cryostability of *bacteria*. A cryoprotectant-free solution of 10% fetal bovine serum was inoculated with *Brucella abortus*, conventionally frozen at a cooling rate of −0.3 °C/min, and later thawed in a 37 °C water bath. It was established that the number of colony-forming units of *B. abortus* in 1 mL of frozen suspension was reduced from 213 million to only 70 million colonies per milliliter and from 213 to only 83 million colonies per milliliter after 2 weeks and after 6 months of storage in liquid nitrogen, respectively [60].

High cryostability of bacteria was demonstrated in a recent publication regarding freezing–drying (cryoprotectant-free cryopreservation followed by vacuumization) of lactic acid bacteria [61].

Mycoplasma is also a cryostable pathogen [62]. It was established that the developmental rate of *M. equigenitalium* and *M. subdolum* was not diminished by cryoprotectant-free cryopreservation by direct plunging into liquid nitrogen and storage at −196 °C for up to 30 days [62].

M. genitalium is an established cause of sexually transmitted infections. Remnants of positive original specimens were frozen without cryoprotectants and stored at −20 °C for up to 18 months. It was established that the viability rate (DNA load) of these bacteria was 90% [63].

The high cryostability of mycoplasmas was evidenced by a publication regarding freezing–drying (cryoprotectant-free freezing was the first stage of the process) of these microorganisms [64].

Various plastic cryovials and cryostraws allow cryopreservation of any types of cells and tissues without direct contact with liquid nitrogen and with no risk of cross-contamination by different pathogens.

Taking into account all of the data mentioned above, in this review we present two types of cryoprotectant-free cryopreservation of human spermatozoa by direct plunging into liquid nitrogen (vitrification): nonaseptic and aseptic.

52.5 Asepticity as an Obvious Parameter of ART (Including Cell Cooling in Liquid Nitrogen) and Hazard of Cross-Contamination by Pathogens in Liquid Nitrogen

In 1972, Piasecka-Serafin [65] described the possibility of translocation of bacteria from infected semen pellets to sterile ones via liquid nitrogen.

Numerous points of view regarding the risk of cross-contamination during cryopreservation of reproductive cells in liquid nitrogen have been presented [58, 66–81].

In the opinion of Isachenko [82], the following three facts evidence the impossibility of sterilization of liquid nitrogen (one of the methods of sterilization was published by Parmegiani et al. [83]).

Firstly, the bacterium *Deinococcus radiodurans* is extremely resistant to ionizing radiation and ultraviolet light, and is capable of withstanding an acute dose of 5000 Gy of ionizing radiation with almost no loss of viability, and an acute dose of 15,000 Gy with 37% viability. For comparison, 5 Gy can kill a human and 200–800 Gy kills *Entamoeba coli* [84].

Secondly, the colony-forming ability of ultraviolet-irradiated mycoplasma cells has been shown to completely recover after 3 h in the dark [85].

Thirdly, activation (an increase in the viability of viruses) has been shown to begin after 5 min of ultraviolet treatment, and maximal activation was noted 10 min after the beginning of ultraviolet treatment [86].

Bielanski [73] notes that "From a practical point of view, complete sterilization and maintenance of sterility in such a robust system might be a very demanding task, if possible at all. Accordingly, some ubiquitous bacterial agents can be expected in any commercially-produced liquid nitrogen. To this author's knowledge, at the present time, there is no commercial supplier of sterile liquid nitrogen or of a portable device producing liquid nitrogen suitable for assisted reproductive technologies (ART)."

52.6 Technologies for Cryoprotectant-Free Vitrification of Human Spermatozoa Involving Direct Contact with Liquid Nitrogen

Isachenko et al. [43] investigated the ability of sucrose to protect spermatozoa against mitochondrial damage, artificial cryoinduction of capacitation, and acrosome reaction. Spermatozoa were cryoprotectant-free vitrified using three different media: (1) human tubal fluid (HTF; control) medium; (2) human tubal fluid with 1% HSA; and (3) human tubal fluid with 1% HSA and 0.25 M sucrose. From each group, 30-μL suspensions of cells were dropped directly into liquid nitrogen. The cells were thawed by quickly submerging the spheres in human tubal fluid with 1% HSA at 37 °C with gentle agitation. The sperm motility, viability, mitochondrial membrane potential integrity, spontaneous capacitation, and acrosome reaction were investigated. The number of progressively motile spermatozoa was significantly higher in the sucrose-supplemented medium group (57%) than in the controls (19%). The combination of HSA and sucrose (65%) had a stronger cryoprotective effect on the integrity of the mitochondrial membrane potential than HSA alone (33%). It was concluded that cryoprotectant-free vitrification of human spermatozoa with nonpermeating cryoprotectants such as HSA and sucrose can effectively cryopreserve the cells without significant loss of important physiological parameters [43].

Two principally different methodologies were compared: freezing with permeating cryoprotectants and cryoprotectant-free vitrification [87]. For freezing, a cryovial of sperm suspension with permeating cryoprotectant was cooled in liquid nitrogen vapor at a cooling speed of about −10 °C/min. For vitrification, 30 μL of cryoprotectant-free suspension was dropped directly into liquid nitrogen. It was

established that the rates of DNA fragmentation were not significantly greater in the vitrification (16%) or rapid-freezing (17%) groups than in the control group (12%). The rates of hyaluronan binding were similar in spermatozoa in the control and cryopreserved groups. The authors noted that addition of cryoprotectant for vitrification had a neutral effect on the rates of sperm recovery. It was concluded that cryoprotectant-free vitrification has great potential for human sperm cryopreservation. However, it is not superior to freezing with cryoprotectant [87].

Chen et al. [88] collected spermatozoa from normozoospermic patients and cryoprotectant-free vitrified them with or without addition of sucrose by directly plunging a small volume of suspension into liquid nitrogen. The spermatozoa recovery rate, motility, viability, chromatin damage, and DNA fragmentation were assessed. No significant differences were observed in the spermatozoa recovery rate and motility rate between spermatozoa that were cryoprotectant-free vitrified without or with sucrose. However, spermatozoa vitrified without sucrose had greater viability and less damage to sperm chromatin and DNA than those vitrified with sucrose [88].

High effectiveness of cryoprotectant-free vitrification was shown in experiments by Agha-Rahimi et al. [89]. Sperm suspensions were divided into four aliquots: fresh sample (control); cryoprotectant-free vitrification in human tubal fluid medium supplemented with 5 mg/mL HSA and 0.25 M sucrose; patients' seminal fluid; and artificial seminal fluid. Vitrification and warming were performed according to the method described by Isachenko et al. [43]. Twenty-five-microliter aliquots of sperm suspension were dropped directly into a strainer that was immersed in liquid nitrogen, using a micropipette. After warming, sperm parameters of motility, viability, and morphology (assessed with light and transmission electron microscopy observations) were analyzed. It was concluded that seminal fluid in normal semen can act as a cryoprotectant and that cryoprotectant-free vitrification of human sperm in artificial seminal fluid can more effectively preserve the spermatozoa than vitrification in human tubal fluid supplemented with HSA and sucrose [89].

52.7 Basis for Cryoprotectant-Free Vitrification of Human Spermatozoa with Full Isolation from Liquid Nitrogen (Aseptic Technology)

In experiments [90] assessing vitrification by direct plunging into liquid nitrogen, the prepared spermatozoa were loaded onto copper loops 5 mm in diameter by dipping the loops into a sperm suspension to obtain a thin film (supported by surface tension) of 20 ± 2 μL. The volume of the sperm suspension included in the film was determined in the following way. After the loop was plunged into the suspension of spermatozoa and a film was formed, the suspension was shaken down onto the flat surface of a plastic petri dish. Then, using a micromeasurer, the researchers were able to determine the volume of the film, which had formed a droplet on the flat surface of the petri dish. The potentially negative effect of the copper on the spermatozoa was ignored, given their short contact with this material. The loaded loops were then plunged into liquid nitrogen. After storage for a minimum of 24 h, the samples were thawed by plunging the loops into a 15-mL tube containing 10 mL of culture medium at 37 °C under intense agitation. After warming of five loops in one tube, the tube was placed in a CO_2 incubator for 5–10 min. Next, the spermatozoa were concentrated by centrifugation at 380 × *g* for 10 min and processed for further evaluation.

For vitrification in liquid nitrogen vapor, the spermatozoa were vitrified and thawed according to the procedure described above, with the following modifications. Before being plunged into liquid nitrogen, the loops were cooled for 3 min in liquid nitrogen vapor at −160 °C. This was achieved by placing the loops in a Styrofoam box containing a 0.5–0.8-cm depth of liquid nitrogen, 1 cm above the liquid nitrogen level. The temperature of the vapor was determined using an electric thermometer.

The speed of cooling during the direct plunge into liquid nitrogen was calculated by introducing variables such as the geometry of the loop, the amount of attached material, and the physical characteristics of the sperm suspension [90]. The speed of cooling of the sperm suspension film on a loop frozen in liquid nitrogen vapor was determined using a method designed by the authors [90]. A loaded loop was placed in the same position in the same box containing liquid nitrogen in which the experimental spermatozoa were frozen. The film was then periodically (at 1-s intervals) pierced by a thin (27-gauge) needle at different locations (in the center, near the copper ring, and at the periphery). When the film was liquid, it was possible to punch through it many times without disruption of the film, and after the needle was removed, the film remained intact. Upon freezing, the film solidified (starting from the copper ring area and extending toward the center) and piercing without disruption of the film became impossible; the ring began to move. The time elapsed (visually indicated) from placing of the loop in the box at room temperature (23 °C) to the beginning of solidification of the suspension (−4 °C) allowed calculation of the speed of cooling of the spermatozoa.

The time that elapsed from when the suspension was at room temperature (23 °C) to the beginning of the suspension film solidification (when the ring began to move at −4 °C) depended on the place where the film was pierced by the needle: from 6 s (with piercing near the copper ring) to 10 s (with piercing at the center of the ring). Thus, the rate of cooling of the spermatozoa in liquid nitrogen vapor was in the range of 162–270 °C/min.

Both cryopreservation regimes caused about a 40% reduction in the motility of the spermatozoa in comparison with the swim-up-treated control. No statistically significant difference in this parameter between the two methods of cryopreservation was found.

In contrast to motility, the DNA integrity in both groups of cryopreserved spermatozoa was found to be unaffected by the vitrification mode. Instead of motility, three parameters of the viability of the spermatozoa after vitrification with rapid and slow cooling, compared with the control (fresh samples), were taken into account: the fertilization rate (formation of pronuclei), early cleavage of zygotes (formation of 4–6 blastomeres), and late development of embryos (formation of a blastocoel). The results of in vitro fertilization (IVF) demonstrated the approximately equal fertilization potential of the human spermatozoa that were cryoprotectant-free vitrified and frozen in liquid nitrogen vapor.

In conclusion, these results point to the feasibility of cryoprotectant-free cryopreservation of human spermatozoa by fast or relatively slow cooling, achieved by direct plunging into liquid nitrogen (vitrification) or freezing in liquid nitrogen vapor beforehand, followed, in both cases, by rapid thawing. The DNA integrity of sperm cryopreserved using both regimes was comparable to that of fresh sperm [90].

The results of the described experiments provided the grounds for creation of aseptic technology for cryoprotectant-free vitrification of human spermatozoa (with full isolation of the spermatozoa from liquid nitrogen).

52.8 Technologies for Cryoprotectant-Free Vitrification of Human Spermatozoa with Isolation from Liquid Nitrogen (Aseptic Technologies)

In experiments performed by Isachenko et al. [42], ejaculate from patients with oligoasthenoteratozoospermia was prepared using the swim-up method and divided into equal parts for vitrification using droplets, open pulled straws, and open standard straws, as well as one fresh (control) group.

The method used to cool the droplets in liquid nitrogen vapor was previously described by Dinnyes et al. [91] for vitrification of oocytes and embryos; however, in the present experiments, a massive metal block was not used. Aliquots of 40 μL of sperm suspension were placed on aluminum foil that had previously been cooled to −160 °C in liquid nitrogen vapor. During cooling, each droplet of sperm suspension adopted a spherical form. After 5 min of cooling, these solidified droplets of sperm suspension were placed into cryovials that had been precooled in liquid nitrogen, for subsequent storage in liquid nitrogen until the time of warming.

To cool the open pulled straws in liquid nitrogen, aliquots of 5 μL of sperm suspension were drawn inside the end of the open pulled straws by capillary action [92]. The straws were placed inside 0.5-mL insemination straws. The 0.5-mL straws were hermetically closed and plunged into liquid nitrogen, using the method described by Isachenko et al. [42].

To cool the open straws in liquid nitrogen, 1-μL aliquots of sperm suspension were deposited inside the ends of standard 0.25-mL insemination straws. The straws were placed inside 0.5-mL insemination straws. The 0.5-mL insemination straws were hermetically closed and plunged into liquid nitrogen, using the method described by Isachenko et al. [42].

To warm the droplets, the samples were thawed by being plunged into a 15-mL tube containing 10 mL of culture medium at 37 °C under intense agitation. After warming (with one droplet in one tube), the tubes were placed in a CO_2 incubator for 5–10 min at 37 °C. Next, spermatozoa were concentrated by centrifugation at 380 × *g* for 5 min, and the resulting pellet was resuspended in 100 μL of culture medium and used for further evaluation.

To warm the open pulled straws and open straws, these straws (after being expelled from the 0.5-mL insemination straws) were rapidly plunged into 1.5-mL microcentrifuge tubes with culture medium at 37 °C. After warming, the spermatozoa were expelled into a petri dish and were then cultured.

The three cryopreservation regimes resulted in approximately 40% reductions in sperm motility in comparison with the swim-up control. Although progressive motility was much reduced just after thawing, the motility of the spermatozoa was increased dramatically 2 and 5 h later, but it decreased again after 24 h of culture. No statistically significant differences in this parameter between the droplet, open pulled straw, and open standard straw cryopreservation regimes were found. Thus, there were no differences in the motility rates of spermatozoa vitrified using these methods during culture periods of 2, 5, and 24 h.

In conclusion, evaluation of two parameters—motility and long-term survival of spermatozoa—suggests that the three described methods are suitable for use in ART.

However, the open pulled straw and open straw methods of vitrification are preferable because they allow isolation of the spermatozoa from the liquid nitrogen, with a maximum reduction in the potential risk of microbial contamination, as described by Isachenko et al. [42].

The aims of the study by Isachenko et al. [93] were to develop and test the aseptic technology of cryoprotectant-free vitrification of human spermatozoa in large volumes (for intrauterine insemination). Each of the 52 swim-up-prepared ejaculate samples was divided into three aliquots and allocated to one of three treatment groups: group 1 was a nontreated control group; group 2 consisted of spermatozoa cryopreserved by slow conventional freezing with glycerol-containing medium, and group 3 consisted of spermatozoa vitrified in 0.5-mL insemination (“french”) straws in culture medium with 0.25 M sucrose. Sperm motility at 1, 24, and 48 h after warming; plasma membrane integrity; and capacitation-like changes (spontaneous “cryocapacitation” and acrosome reaction) were assessed after freezing–thawing. Prior to vitrification, the spermatozoa were processed by the swim-up technique and thereafter diluted 1:1 with 0.5 M sucrose. The final concentration of sucrose prepared with bidistilled water was 0.25 M. The packaging of spermatozoa for aseptic vitrification was performed in the following way. Spermatozoa suspensions were cooled in 0.5-mL plastic straws. The hermetically sealed straws were

immersed in liquid nitrogen. The warming-up of the spermatozoa was achieved by immersing the straws with vitrified spermatozoa in a water bath at 42 °C and dangling them gently in the water for 20 s. After warming, the residual fluid was removed from the straws with a paper towel and the straws were disinfected with 70% ethanol. The heat-sealed ends of the straws were cut off with sterile scissors and the suspension was expelled from the straws for immediate evaluation of sperm quality, and for loading into a catheter and intrauterine insemination. It was established that in comparison with conventional freezing, spermatozoa vitrified with aseptic cryoprotectant-free technology displayed superior functional characteristics. The motility rate and the integrity rates of the cytoplasmic and acrosomal membranes were significantly higher after vitrification than after conventional freezing (76% vs. 52%, 54% vs. 28%, and 44% vs. 30%, respectively). However, there were no differences between vitrification and conventional freezing in the presence of glycerol in terms of the percentages of spermatozoa expressing a cryocapacitation pattern (11% vs. 10%). It was concluded that basic protection from cryoinjury can be achieved for human spermatozoa using the novel technology of aseptic cryoprotectant-free vitrification in large volumes. Spermatozoa vitrified with use of this technology are free of permeating cryoprotectants and are ready for further use immediately after warming without the need for any additional treatment (centrifugation or separation in the gradient for removal of cryoprotectants [93, 94].

In investigations by Isachenko et al. [94], a couple, both 39 years old, underwent ART because of severe endometriosis and oligoasthenoteratozoospermia. The spermatozoa suspension was prepared on culture medium supplemented with 1% HSA and diluted (1:1) with 0.5 M sucrose prepared in double-distilled water. After dilution, the cell suspension was maintained at 37 °C in a 5% CO_2 atmosphere for 5 min before being directly plunged into liquid nitrogen.

Cooling of the spermatozoa was performed using a cut standard straw [95]. A 10-μL aliquot of spermatozoa suspension was deposited on the end of the inner part of the straw. Then the straw was placed inside a 0.5-mL insemination straw, which was sealed hermetically with a handheld sealer and plunged into liquid nitrogen.

For warming, the packaged cut standard straw was partly removed from the liquid nitrogen and the upper part of the 0.5-mL straw was cut off. Then, the cut standard straw was removed from the packaged straw and quickly immersed in 1.8 mL of culture medium, which had been prewarmed to 37 °C in a 2-mL centrifuge tube. In this way, very fast warming of the spermatozoa (30,000 °C/min) was achieved.

Next, the warmed and diluted spermatozoa were concentrated by centrifugation and used for further culture, evaluation, and ICSI. The freshly prepared swim-up and thawed vitrified spermatozoa were assessed for changes in the following physiological and morphological parameters: progressive motility, capacitation-like membrane changes, acrosome reaction, and mitochondrial membrane potential.

Eight mature (metaphase II) oocytes were microinjected with the vitrified–warmed spermatozoa. Eighteen hours later, six oocytes showed signs of normal fertilization. Two four-blastomere embryos were transferred to the uterine cavity.

Thirty minutes after thawing, the vitrified spermatozoa displayed 60% progressive motility (vs. 90% in freshly prepared swim-up spermatozoa); 10% were identified as displaying a "cryocapacitation" pattern and 5% were identified as displaying an "acrosome reaction" pattern (vs. 8% and 5% of freshly prepared swim-up spermatozoa, respectively). Sixty-three percent of spermatozoa were classified as having high mitochondrial membrane potential (vs. 96% of freshly prepared spermatozoa). Two healthy girls were subsequently born.

The first case of a healthy baby born after intrauterine insemination with vitrified swim-up spermatozoa from an oligoasthenozoospermic patient was reported in 2012 [84]. A 39-year-old patient underwent intrauterine insemination with spermatozoa from her 35-year-old husband diagnosed with oligoasthenozoospermia. The swim-up spermatozoa from two ejaculate samples were suspended in a culture medium supplemented with 1% HSA and 0.25 M sucrose. Three hermetically packaged 100-μL sperm portions were vitrified by being directly plunged into liquid nitrogen.

Before intrauterine insemination, all portions were warmed in culture medium at 37 °C and the spermatozoa were concentrated by centrifugation; they were then resuspended in 500 μL of culture medium and inseminated. The cell suspension used for insemination displayed 60% progressive motility. Clinical pregnancy was confirmed, and a healthy baby was born at term. These results showed that successful intrauterine insemination can be achieved with aseptic vitrification of spermatozoa [96].

The aims of the study by Isachenko et al. [97] were to develop and test the standardized aseptic technology of permeating cryoprotectant–free vitrification of human spermatozoa in capillaries (for ICSI or IVF in microvolumes). To test the effects of vitrification on basic sperm parameters, 68 swim-up-prepared ejaculate samples from oligoasthenoteratozoospermic patients were aliquoted and distributed into three groups: (1) a nontreated control group, (2) a group consisting of 10-μL samples of spermatozoa cryopreserved by slow conventional freezing with glycerol-containing medium, and (3) a group consisting of 10-μL samples of spermatozoa vitrified in 50-μL plastic capillaries in culture medium with 0.25 M sucrose.

Spermatozoa motility (at 1, 24, and 48 h after warming), plasma membrane integrity, acrosomal integrity, and spontaneous capacitation-like changes were determined after warming.

For preparation of the vitrification solution, the basic medium (culture medium +1% HSA) was diluted 1:1 with 0.5 M sucrose prepared with bidistillate water. The spermatozoa were centrifuged and resuspended with this vitrification solution. For this methodology, 50-μL plastic capillaries were manufactured from hydrophobic material as vehicles for cooling sperm cell suspensions. The capillary was filled

with 10 μL of spermatozoa suspension by aspiration. It was absolutely crucial for the inner surface of the capillary to not become moist during the packaging procedure. The authors noted that aspirating the volume of sperm cell suspension above the mark and correcting it by lowering the fluid level inside the capillary after aspiration was technologically wrong and would result in an excess volume after thawing. After aspiration was completed, the capillary was inserted into a 0.25-mL insemination straw. After sealing of both ends, the straw was plunged into liquid nitrogen and cooled at 600 °C/min.

For warming, the capillary was removed from the isolating 0.25-mL insemination straw and immersed in a 1.8-mL centrifuge tube with 0.7 mL of vitrification medium (culture medium with sucrose) prewarmed to 37 °C. The authors noted that the volume of vitrified suspension after warming was not increased. Finally, the suspension of spermatozoa was expelled from the capillary for immediate evaluation of spermatozoa quality and use in IVF or ICSI.

It was established that aseptic cryoprotectant-free vitrification showed a significantly stronger cryoprotective effect than conventional freezing. One hour after warming, the rates of motility, plasma membrane integrity, and acrosomal integrity were significantly higher than those observed in conventionally frozen spermatozoa (28% vs. 18%, 56% vs. 22%, and 55% vs. 21%, respectively) but lower than those observed in fresh spermatozoa (35%, 96%, and 84%, respectively). The rates of capacitation-like changes did not differ significantly between vitrified and conventionally frozen samples (8% vs. 9%) but were higher than the rate observed in fresh spermatozoa (2%).

It was concluded that the newly developed technology of aseptic vitrification of human spermatozoa in capillaries can effectively preserve these cells from cryoinjuries [97].

The following nonaseptic technology was used in experiments by Khalili et al. [98]. The aim of the investigations was to evaluate the effects of neat semen vitrification on vital parameters and DNA integrity of sperm from men with normal and abnormal sperm parameters.

The semen was loaded onto copper cryoloops 2.5 mm in diameter by dipping the loops in the suspension to obtain a thin film, and the loaded loops were plunged into liquid nitrogen. After storage for 7 days, the samples were warmed by plunging the loops into a tube containing 2.5 mL of culture medium at 37 °C. After warming of ten loops in one tube, the tube was placed in a CO_2 incubator for 5–10 min. Then the spermatozoa were centrifuged at 300 × *g* for 10 min, and the resultant pellet was resuspended in 100 μL of culture medium and processed for further evaluation. It was established whether each semen sample was a normozoospermic sample or a specimen with abnormal sperm parameters.

Cryoprotectant-free vitrification of the spermatozoa resulted in significant decreases in sperm motility, viability, and normal morphology after thawing in both normal and abnormal semen. Also, the rates of sperm DNA fragmentation were significantly higher in the vitrified samples than in the fresh samples in both the normal semen group (24% vs. 16%) and the abnormal semen group (34% and 23%). It was concluded that vitrification of neat ejaculate has a negative impact on both sperm parameters and DNA integrity, particularly among abnormal semen samples. The authors therefore recommended processing semen samples before vitrifying the sperm pellets [98].

The following investigation by Kuznyetsov et al. [99] used technology that could also be characterized as aseptic. The purpose of this study was to optimize cryoprotectant-free vitrification and post-thaw recovery of a small number of spermatozoa in a closed straw system in normozoospermic and severely oligozoospermic samples. Spermatozoa in 5-μL droplets of a cryoprotectant-free vitrification medium were placed in open pulled straws [92], which were then inserted into 0.5-mL insemination straws. The insemination straws were hermetically closed at both ends and plunged into liquid nitrogen. For warming, the end of each insemination straw was cut with scissors and the open pulled straw was removed with forceps. The open narrow end of the open pulled straw with vitrified spermatozoa was immediately immersed in culture medium for 10 s. The capillary flow of the warm medium allowed melting of the vitrified sample. Subsequently, the content of the open pulled straw was expelled, with the help of a syringe, into a droplet of media, and each sample was assessed to confirm the number of recovered spermatozoa and sperm motility. The overall sperm recovery rate after vitrification was 80%, with 80% viability and 42% retained postwarming motility [99].

A study by Slabbert et al. [100] aimed to compare cryoprotectant-free vitrification and conventional freezing protocols for spermatozoa cryopreservation. Washed samples of spermatozoa were split into two aliquots and cryopreserved by cryoprotectant-free vitrification or conventional freezing with glycerol.

Spermatozoa were vitrified without permeating cryoprotectants by Isachenko et al. [93] in culture medium supplemented with HSA and 0.25 M sucrose with use of 0.5-mL straws. After filling of the straws with spermatozoa suspension, the straws were hermetically closed and plunged into liquid nitrogen. The mitochondrial membrane potentials and DNA fragmentation were evaluated. No significant differences were observed in the sperm motility parameters. A significantly higher mitochondrial membrane potential percentage (12% vs. 7%) and a lower DNA fragmentation percentage (3% vs. 4%) were observed in spermatozoa after cryoprotectant-free vitrification and after freezing with glycerol, respectively. The authors concluded that cryoprotectant-free vitrification is a rapid and promising alternative to conventional cryopreservation using cryoprotectants. For thawing, the straws were immersed in a water bath at 42 °C for 20 s, in accordance with the protocol suggested by Isachenko et al. [93]. It was concluded that cryoprotectant-free vitrification of spermatozoa provides a simpler, faster, more cost-effective alternative to conventional cryopreservation methods [100].

Ali Mohammed [101] compared conventional freezing of human spermatozoa in 0.25-mL insemination straws with permeating cryoprotectant and cryoprotectant-free vitrification in a culture medium with 1% serum supplement and 0.25 M sucrose. For vitrification, 0.25-mL insemination straws were filled with 100 μL of the sperm suspension and placed in 0.5-mL insemination straws, which were hermetically closed at both ends and plunged directly into liquid nitrogen. For warming of the vitrified probes, the ends of the 0.5-mL straws were cut and the 0.25-mL straws were taken out and inserted into 15-mL plastic tubes containing 5 mL of culture medium prewarmed to 42 °C. The viability and mitochondrial membrane potential of the spermatozoa were assessed. Both cryopreservation techniques yielded similar results. However, vitrification was faster, easier, less expensive, and associated with less toxicity [101].

Mansilla et al. [102] determined the optimal temperature of warming for vitrified human spermatozoa. After centrifugation of the spermatozoa, the pellet was diluted with 1% dextran + 0.5 M sucrose. Then 100 μL of sperm suspension was packaged into 0.25-mL insemination straws. Each 0.25-mL straw was horizontally inserted inside a 0.5-mL insemination straw, which was then immersed in liquid nitrogen. After warming, sperm motility and the functionality of the sperm membranes were evaluated. It was detected that the progressive motility rates of sperm warmed at 38, 40, and 42 °C were 26%, 57%, and 65%, respectively. The plasma membrane function was better preserved by thawing at 42 °C (76%) than at 40 °C (43%) or 38 °C (65%). It was concluded that warming of the vitrified spermatozoa at 42 °C with 5 s of exposure in the thawing medium preserved highly motile cells and plasma membrane function [102].

52.9 Conclusions

The present data show extremely high viability of viruses, bacteria, mycoplasmas, and fungi after cryoprotectant-free cryopreservation in culture medium and even in distilled water. During vitrification, spermatozoa should be isolated from liquid nitrogen, and a number of such aseptic technologies have been described.

Competing interests The authors declare that they have no competing interests.

Review Questions

1. What is known about microvibration in general?
2. Is vibration a natural phenomenon?
3. Does vibration have a stimulating effect on living systems?
4. Does vibration play a role in mechanical transduction?

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Slow Freezing of Oocytes

Giovanni Coticchio and Lucia De Santis

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Learning Objectives

- Importance of oocyte cryopreservation programs in female fertility management.
- Importance of rigorous management systems to control cryopreservation protocols.
- Pros and cons of slow-freezing procedures.
- Objective viability evaluation of slow-cooled oocytes compared to vitrified counterparts.
- Cytoskeletal and ultrastructural assessment of slow-cooled oocytes.
- Clinical outcomes of cycles (IR, PR, and CPR) with respect to different CRSC protocols.

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53.1 Introduction

Assisted reproduction technology (ART) is becoming an increasingly important strategy to preserve female fertility. Until a decade ago, in an ART context, the only option available to preserve fertility for a woman at risk of loss of ovarian function was embryo cryopreservation. The storage of embryos is a sound, well-established, and safe procedure but entails major downsides, such as ethical objections to the generation of conceptuses that might never be used, the need of donor sperm for the treatment of single women, and legal complications in case of separation of the couple under treatment. More recently, new avenues have been opened to preserve female fertility through ART. Ovarian tissue cryopreservation and orthotopic reimplantation have shown the potential to restore ovarian function for a period of time and offer a chance for natural conception. However, so far only a limited number of full-term pregnancies have been achieved [1]. Therefore, the efficacy of this approach remains to be established. Oocyte cryopreservation is powerfully established as an efficient ART procedure able to preserve female fertility without the implications that afflict embryo cryopreservation. Historically, control rate slow cooling (CRSC) has been the technological standard of human embryo cryopreservation. This legacy marked the early history of oocyte cryopreservation. In fact, specifically developed CRSC protocols gave oocyte cryopreservation the dignity of a routine procedure in many human IVF laboratories. Cryopreservation of slow cooling remains nowadays a viable option, although progress in the last several years has been mainly driven by vitrification (Box ► 53.1).

Box 53.1 Optimized slow freezing protocol for oocyte cryopreservation. From Bianchi et al. (2007)

Materials

- PBS (Dulbecco's phosphate-buffered saline)
- PPS (Plasma protein solution, 5% g/v) or other protein supplement
- Propane-1,2-diol (PrOH)
- Sucrose
- 4-well plates
- Freezing straws
- 1 ml syringes

53.2 Methodological Aspects of Oocyte Cryopreservation

Oocyte cryopreservation, irrespective of whether it is achieved by CRSC or vitrification, requires a rigorous management system to control technique (type of protocol, cryopreservation media) and procedure (time of oocyte culture before and after cryopreservation, oocyte manipulation, operator experience) that could affect the viability of the stored material. In terms of operative times and mobilization of resources, cryopreservation can represent a conspicuous part of the activity of an IVF laboratory. Therefore, cryopreservation and long-term storage in liquid nitrogen necessitates specific work areas. These areas should be adjacent to the core of the laboratory, accessible only by authorized staff, and equipped with systems to monitor oxygen levels and ensure sufficient air changes to prevent accumulation of nitrogen vapors. An approach to cryopreservation based on CRSC should be supported by the use of two cryofreezers, to ensure safe backup and give the opportunity to run separate cycles of freezing at appropriate times on the same day, if required. Cryofreezers produce temperature graphs of the cooling process that represent an important document of quality control for each cryopreservation cycle. Liquid nitrogen levels in dewars used for medium-/long-term storage should be monitored at regular intervals and reported in a dedicated logbook. More recently, dewars have been equipped with electronic monitoring systems to be able to send information to remote terminals, such as PC and mobile phones. Laboratory staff should be properly instructed on safety measures concerning the use of liquid nitrogen and have access to devices for personal protection. Quality control and quality assurance programs require monitoring and recording of the procedures adopted for the cryopreservation of oocytes. These data may be used at later stages for various purposes, especially to assess efficacy and reproducibility. Each laboratory should have standard operating instructions describing the cryopreservation protocols and flow charts showing the path that oocytes follow from recovery to their use after cryopreservation. All aspects pertaining to cryopreservation procedure (information for the identification of the couple, cryopreservation protocols, storage details) should be fully described and reported in a logbook and an electronic database backed up at regular intervals.

53.3 Possible Effect of Slow Freezing in Human Oocytes

Fully grown oocytes from antral follicles may be cryopreserved at the immature—germinal vesicle (GV)—or mature stage. Immature oocyte cryopreservation has been suggested to represent a possible route for fertility preservation especially in women facing the prospect of gonadotoxic therapies [2]. This option was inspired from the hypothesis that the risk of chromosome aberrations possibly associated with the process of freezing-thawing could be attenuated by the fact

that in immature oocytes chromosomes are protected inside the nucleus and the cytoskeleton is not organized in a meiotic spindle. Therefore, under these conditions, chromosome integrity and segregation should not be affected. However, recent data have shown that other important cellular structures of the immature oocyte and companion somatic cells may be subject to damage as an effect of cryopreservation. In particular, cryopreservation conditions cause physical disruption of trans-zonal projections (TZPs), axon-like microfilament-rich projections that originate from cumulus cells, cross the zona pellucida, and make contact with the oolemma [3, 4]. In the sites of contact, cumulus cells communicate with the oocyte by means of gap junctions [5]. Gap junctions are critical for the transport of small size [less than 1 kDa] cumulus-derived factors, metabolites, and signaling molecules that are necessary for immature oocytes to resume meiosis and acquire full cytoplasmic maturation and developmental competence. Therefore, the documented damage to TZPs offers a plausible explanation of the poor performance of oocyte cryopreserved at the GV stage, in terms of maturation, fertilization, and embryo developmental capacity [6–8]. Moreover, recent data describing the redistribution of mRNAs and cytoskeletal proteins during GV to MII transition confirm the importance of full maturity to support proper fertilization and development [9].

The storage of mature oocytes has gained a far larger interest as an alternative to embryo cryopreservation. The preovulatory mature oocyte is an individual entity, ready for fertilization and relatively autonomous from the cumulus cells that have accompanied and supported its development throughout oogenesis. Its cryopreservation, therefore, is not complicated by the need to maintain unaltered a sophisticated tissue and intricate cell contact, as in the case of the preservation of ovarian tissue. Nevertheless, the cellular and biochemical integrity of the single oocyte is exposed to a risk of irreversible damage during the process of cryopreservation. Rupture of the oolemma and widespread cell disruption is a relatively common event. This occurs in 20−30% of cases even with the most efficient CRSC protocols [10, 11], a frequency that has become less acceptable following the recently reported successes of the vitrification approach by which survival rates in excess of 90% can be achieved. Oocyte death may be observed immediately after freezing and release from the storage device (usually a 250 µl straw) or during exposure to thawing solutions. Clearly, this may represent an effect of physical damage caused by intracellular ice formation or osmotic stress dictated by dehydration and rehydration events taking place during replacement of intracellular water with cryoprotective agents (CPAs) and/or vice versa [12]. Cell death may occur even hours after the completion of the process of thawing and rehydration. Gook et al. [13] reported a decrease in survival rate of oocytes over the 24 h following thawing. Clearly, oocyte cryopreservation by CRSC requires further development in order to improve survival rates.

Survival after thawing is obviously crucial, but does not necessarily ensure or coincide with cell integrity. Sublethal damage may affect different cell organelles and structures and/or biochemical pathways. Transmission electron microscopy (TEM) is an investigative tool often believed either obsolete or out of fashion by many, but in fact, it is extremely valuable for the study of cell damage that may be generated by cryopreservation. In human oocytes stored by a CRSC protocol developed for human embryos, Sathananthan observed fractures in the zona pellucida, oolemma irregularities, and widespread disorganization of the ooplasm. After exposure to dimethylsulfoxide (DMSO) at 0 °C, he also found that parts of the endoplasmic reticulum, Golgi, mitochondria, and the cytosol were damaged to some extent [14]. A subsequent study showed how sometimes TEM fails to detect structural anomalies that may have significant implications for the oocyte physiology. Van Blerkom and Davies [15] described cytokinesis anomalies and cleavage arrest in embryos developed from cryopreserved human oocytes, despite the fact that electron microscopy analysis carried out after thawing had not shown gross cytoplasmic alterations. Afterward, electron microscopy studies on cryopreserved human oocytes were interrupted for over a decade, as a consequence of the inadequacy of the cryopreservation methodology available in the 1990s to ensure high survival rates. With the development of improved protocols [11, 16, 17], ultrastructural studies again attracted significant interest. By comparing fresh and frozen-thawed oocytes, Ghetler et al. [18] reported substantial decrease in the number of cortical granules (CG) as an effect of cryopreservation. This supports the inference that stored oocytes should be microinjected by intracytoplasmic sperm injection (ICSI) rather than inseminated by standard IVF to prevent possible fertilization failure caused by hardening of the zona pellucida. In effect, a few years after its introduction as a routine fertilization method, ICSI was chosen as the elective route to achieve fertilization in cryopreserved oocytes [19] and is now recognized as a standard of treatment involving cryopreserved oocytes [20–23]. Despite that, the question of a non-physiological release of CG in cryopreserved oocytes remains open. By using epifluorescence microscopy, Gook et al. [13] showed that, in comparison to fresh material, the staining specific for CG was unaffected in cryopreserved material. They also showed that in frozen oocytes, fertilization could be achieved through standard IVF [24]. The possibility of using standard IVF has been reported by other authors [25]. However, further ultrastructural analysis has confirmed that a partial loss of CG occurs following cryopreservation. This has been observed qualitatively in oocytes cryopreserved with CRSC protocols involving the use of 0.1 or 0.3 mol/l sucrose as a constituent of the freezing solution [26]. Similar findings were described by Gualtieri et al. [27]. Consistent with this, oocytes stored by a CRSC protocol including ethylene glycol (EG) as an intracellular CPA [28] or by vitrification [29] appear to be affected by a loss of CG. Recent observations provide quantitative data suggesting that about two-thirds of the original population of CG is lost and presumably their contents released in the perivitelline space, as a result of cryopreservation. This phenomenon appears therefore rather ubiquitous in cryopre-

served oocytes, irrespective of the cryopreservation approach (CRSC or vitrification) or specific protocol. Whether CG release is always sufficient to cause zona hardening in frozen oocytes is not clear, but the detection of thickening and change in texture of the inner surface of the ZP is in line with this hypothesis [28].

Another recurrent characteristic found in cryopreserved oocytes is the increased presence of vacuolar structures. The nature of these formations has not been well characterized. They may be found in fully grown immature oocytes, but in metaphase II (MII) oocytes, they are rare [30] and have been interpreted as a sign of cytoplasmic immaturity or, vice versa, aging. Because vacuoles may also derive from a general response to injury, in the case of oocyte cryopreservation, they may represent a manifestation of cell stress. Rather consistently, the presence of vacuoles was described in association with a variety of CRSC protocols [18, 26, 28]. It is tempting to hypothesize that vacuoles located peripherally may evolve from crypt-like invaginations and clusters of endocytic vesicles that form in the oocyte cortex following simple exposure to CPA [31]. However if this assumption is correct, it remains to be explained why oocytes vitrified through the cryoleaf method, which are exposed to high concentrations of CPAs, do not exhibit an increase in the number of vacuoles, as recently reported [32]. Regardless of their origin, it is possible that vacuoles may be adopted as a specific indicator of cryodamage and reduced developmental potential in cryopreserved oocyte, considering that an increased number of vacuoles [26] and a reduced developmental ability [20, 33] are concomitantly associated to certain CRSC protocols. This indication, however, has not been confirmed through a prospective randomized control trial. Evidence suggests that vitrification protocols are associated with high implantation ability [34] and a normal or low incidence of vacuoles [32].

Mitochondria and elements of the smooth endoplasmic reticulum may also be affected by cryopreservation. Individually, these two types of organelles play well-characterized roles in all cells. In particular, in oocytes, not only are mitochondria central to the energy generation process but also can produce intermediates of the tricarboxylic acid cycle and reducing equivalents that may be employed for antioxidant defense. The SER complex is instead well characterized as an organelle system competent to the translation, modification, packaging, and delivery of proteins destined to the plasmalemma or exocytotic compartments. In the oocyte, the association between mitochondria and SER elements contributes to the generation of the typical oscillations in cytosolic-free calcium that are triggered by the fertilizing spermatozoon and are interpreted by the oocyte biochemical machinery as a start signal for fertilization [35]. Magnitude and frequency of calcium oscillation have also been found to influence much later events of development, such as organogenesis and fetal growth [36]. For such reasons, mitochondria-SER aggregates are particularly important for the physiology of the oocyte. Mitochondria and SER elements, separately in close association with each other, are often found unaltered in oocytes cryopreserved with CRSC protocols relying on propane-1,2-diol (PrOH) as a intracellular CPA [26] and used in the clinical practice [20, 33]. This is a reassuring evidence in the light of the role of mitochondria-SER associations in the mechanism of intracellular calcium signaling, as discussed above. Conversely, other cryopreservation conditions may be in fact deleterious to mitochondria-SER aggregates. In particular, in oocytes cryopreserved by a CRSC protocol including EG as an intracellular CPA, pronounced disorganization of these aggregates may be noticed [28]. Why some cryopreservation conditions can generate damage to the mitochondria-SER aggregates is still to be understood. It may be relevant to this question the fact that, so far, disarrangements of mitochondria-SER aggregates have been reported only in association with the use of EG, but not PrOH. Perceptible, although moderate, underdeveloped mitochondria-SER aggregates were also observed in oocytes vitrified with a solution containing EG [32]. However, such findings are largely insufficient to conclude that EG can specifically and directly affect the constitution of mitochondria-SER aggregates.

Recently it was reported that oocyte ultrastructural dysmorphisms related to cryopreservation and possibly responsible for low oocyte fertilizability not only occur during freezing and thawing, in a strict sense, but also during post-thaw rehydration [37]. These cellular alterations, induced by low temperatures and by osmotic and chemical forces produced during cycles of dehydration-rehydration as well, may alter the distribution and activity of oocyte cellular components. In particular, although slow freezing appears to ensure a good overall preservation of the oocyte, vacuolization and CG release remain crucial limits. It seems also worth noting that all systems of ooplasmic membranes appear significantly affected by freeze-thawing but, except for CGs, their alterations seem to undergo a partial or, more rarely, an almost complete recovery after thawing, at the end of the rehydration process. In addition, the observed variations in the number of M-SER aggregates and MV complexes, occurring during freeze-thawing, suggest that a dynamic process of transition between these two forms of organelle associations may occur. In this regard, it should not be excluded that vacuole and CG membranes, and oolemma as well, may take part in the recycling mechanism. Such shuttle of membranes, starting during freezing and/or at thawing but mainly occurring during rehydration, may be related to alterations of the cytoskeletal stiffness [38] presumably due to PrOH administration and/or withdrawal [39, 40]. We cannot exclude, of course, that the described membrane restructuring is also related to calcium disturbances. From a merely morphological point of view, this recycling reveals a sort of morphogenetic multipotency of the oocyte cytomembranes, possibly eliciting membrane turnover and delivery or clearance of substances (CG content, cryoprotectants, calcium, other solutes?), as postulated for other cells [41].

Overall, these studies confirm the usefulness of TEM to investigate oocyte quality after cryopreservation.

53.3.1 In Vitro Aging

One of the factors that could cause a partial loss of oocyte viability after cryopreservation is the phenomenon of in vitro aging. In fact, only for a limited period of time, the mature oocyte is able to maintain a particular condition that is essential for successful fertilization and development. Such a condition corresponds biochemically to relatively high levels of maturation promoting factor (MPF) and microtubule-activated protein kinase (MAPK), two key regulators of the meiotic and mitotic cell cycles, and from a cellular standpoint to the arrest of the meiotic process at the MII stage. The MPF and MAPK activities can decrease either as an effect of a spontaneous biochemical decay occurring within a few hours after PBI extrusion or secondarily to extrinsic factors, such as inappropriate manipulation in vitro. Cryopreservation could interfere with the mechanism of meiotic control of the oocyte, either directly, through the stresses generated by the process of freezing-thawing (or vitrification-warming), or indirectly, because of an excessively protracted maintenance in culture before and/or after cryopreservation. Recent evidence suggests that biochemical cryodamage is not purely hypothetical. In fact, in sheep, oocytes cumulus removal and vitrification can affect the MPF and MAPK levels after in vitro maturation [42]. A similar phenomenon may occur in human oocytes after cryopreservation. In coincidence with the use of a protocol involving 0.3 mol/l sucrose as non-penetrating CPA [11], over a period of 2 h after thawing, MAPK is unaffected in comparison to fresh controls. Conversely, during the same interval, MPF activity is initially maintained unaltered but undergoes a significant decrease thereafter [43]. It is striking that the observed decrease in MPF mirrors a progressive loss in the organization of the MII spindle that is known to occur with a similar dynamic in frozen-thawed oocytes [44]. Because high levels of MPF are critical to maintain the integrity of the MII spindle, it is tempting to speculate that after thawing a decrease in the activity of the former may cause a loss in the organization of the latter.

These findings may have significant implications for the clinical use of frozen-thawed oocytes. In particular, considering the observed delayed reduction in MPF activity, it might be appropriate to limit to 1 h the period of post-thaw culture that is implemented in preparation for ICSI, thereby preventing possible losses in spindle and chromosome configuration or premature exit from the MII arrest. The importance of the time factor in oocyte cryopreservation seems to be suggested also by data that indicate that the oocyte ability to give rise to a viable pregnancy tends to decrease if the overall time spent in culture before and after cryopreservation exceeds a certain number of hours (Borini et al., unpublished observations). Other recent studies appear to confirm that an excessively protracted period of culture between retrieval and cryopreservation may have a negative impact on the developmental ability of frozen-thawed human oocytes [45, 46]. However, the problem of in vitro aging does not seem to concern oocytes cryopreserved by vitrification, at least in donor oocyte cycles. In fact, Vassena and colleagues showed that fertilization and other developmental parameters were unaffected by a number of parameters, including the time between pickup and vitrification and between warming and microinjection [47].

53.4 Clinical Outcome of Oocyte Cryopreservation

Cryopreservation strategies can profoundly affect the clinical outcome of oocyte cryopreservation and make difficult the comparison among different studies. In certain contexts, constraint of religious, ethical, or legal nature limits the number of embryos that may be produced and transferred during each cycle of treatment. In frozen-thawed oocyte (or vitrified-warmed) cycles, these restrictions can be met by thawing only a small proportion of the stored material at a time to the end of having 2–3 viable oocytes suitable for insemination, avoiding wastage of material. As an effect of a legal restriction implemented in Italy from March 2004 to April 2009, the practice established by law to thaw a limited number of oocytes for treatment cycle was adopted systematically and described in various studies [33, 45, 48]. In other studies, no limit was imposed on the number of oocytes that could be thawed and, consequently, the number of embryos that could be cultured and transferred [49, 50]. The different implications of these two strategies are rather obvious. The thawing of only a few oocytes per cycle involves a higher risk of drop out of the frozen cycle as an effect of high rates of fertilization or cleavage failure [20]. Furthermore, with only a few oocytes available, embryo selection is not applicable, and in some cases, the number of embryos available for transfer may be insufficient. This scenario, which is unlikely to occur when several oocytes are thawed at the same time, influences the clinical outcome in terms of implantation and pregnancy rates. For this reason, the comparison of implantation rates achieved in studies in which the mean number of transferred embryos was different appears rather arbitrary and non-informative [20, 51]. An example of the inadequacy of pregnancy rate per transfer as a measure of efficacy is offered by a study [48] in which by using a freezing protocol specific from cleavage stage embryo, a success rate of 16.7% was achieved. In fact, in the same study, the pregnancy rate per thawing cycle was 7.7%, as a consequence of a high incidence (50%) of cycles cancelled for failed survival or fertilization. Gook and Edgar [52, 53] proposed a more objective standard to assess the efficacy of treatments involving frozen oocytes or embryos. By considering that during an IVF treatment the initial amount of biological material (oocytes) undergoes a decrease (attrition) at different stages of the process (fertilization, cleavage, selection for freezing, and thawing), they estimated that from an original pool of 100 fresh oocytes, about five implantations from frozen embryos can be achieved. Gook and Edgar suggested that the same approach

should be adopted also for the assessment of the clinical efficacy of oocyte cryopreservation, including in the calculation all the events of pre- and post-storage loss of the original material. Only under those conditions the relative values of the diverse cryopreservation methods can emerge. For example, it is well known that a considerable increase (35–40% to 70–75%) in the survival rate oocytes cryopreserved by CRSC may be obtained by increasing from 0.1 to 0.3 mol/l the concentration of sucrose in the freezing solution [3]. A higher sucrose concentration is also beneficial to the fertilization rate [33, 48]. However, the reduced loss of material during the thawing and fertilization phases is accompanied by a decreased implantation rate in comparison to the outcome derived by the use of the protocol based on 0.1 mol/l sucrose [20, 48]. Therefore, in the final analysis, the two protocols result equivalent if assessed on the number of implantations (approximately 2.4–2.6 in both cases) that can be obtained from 100 thawed oocytes. By using other protocols, implantations rates per thawed oocytes of about 5% can be achieved, a result that makes oocyte freezing rather comparable to embryo freezing. By adopting a freezing protocol based on sodium-free media, Boldt et al. [50] reported an implantation rate per oocyte thawed of 5.3%. In that study, however, only 23 patients were included and the initial rate of success has not been confirmed by the treatment of larger series of patients. A comparable implantation rate per thawed oocyte has been reported in another study reported by Borini and colleagues [10], in which oocytes were cryopreserved with a protocol based on different sucrose concentrations in freezing and thawing solutions (0.2 and 0.3 mol/l, respectively). More recently, the same group confirmed these results reporting a success rate of 5–6% implantation rate per thawed oocyte (with obvious differences depending on female age) in a much larger study involving almost 350 patients [54]. Using such differential sucrose concentrations during freezing (dehydration) and thawing (rehydration) seems to be the silver bullet to achieve maximum performance of CRSC in oocyte cryopreservation (see enclosed protocol). In fact, with this protocol, in women younger than 38 years of age, Gook and Edgar obtained a rate of implantation per thawed oocyte of 6.3% [55]. Importantly, both in this study and that of Borini and colleagues [10], rates of fertilization, cleavage, and implantation of embryos developed from frozen oocyte were comparable to those observed in fresh control oocytes. The study of Gook and Edgar [55] was notable also because it assessed the impact of female age in oocyte cryopreservation. The inverse relationship between female age and embryo implantation potential is well known and represents the major cause of reproductive failure in women of advanced reproductive age. Consistently, Gook and Edgar observed a reduced implantation potential in embryos developed from frozen oocyte recovered from women older than 38 years of age. However, the difference in implantation rate between the groups of younger and older women was more than six fold (6.3% vs. 1.0%), a hiatus difficult to explain with the exclusive impact of female age. Rather, it is more plausible that oocytes of older women are particularly sensitive to cryodamage, which acts additively to female age to produce a drastic reduction in plantation rates. Consistent with these data produced by CRSC is a study of Cobo and colleagues [56], who reported implantation rates of 6.6% and 1.8% for embryos developed from vitrified oocytes collected by women with age <36 or >39, respectively. Therefore, oocytes of older women are particularly exposed to cryodamage, irrespective of the cryopreservation approach.

53.5 Safety of Oocyte Cryopreservation

Oocyte cryopreservation has raised health concerns derived from the possibility that oocyte cell damage secondary to freezing-thawing is a frequent event that may expose the conceptus to an increased risk of developmental anomalies. The number of babies born from cryopreserved oocytes is steadily increasing. In 2004, Borini et al. [20] described 11 births from cryopreserved embryos. These children had an average weight of 3.2 kg, normal karyotype, and no malformations. Shortly afterward, Chen et al. [49] reported the birth of five children from vitrified oocytes with normal karyotypes. Other authors [10, 21, 33, 45, 48, 50, 57] documented 65 births from frozen oocytes but did not describe details on their genotype in a systematic fashion. In the 18 births of Levi Setti et al. [21], a mean gestational age of 37.1 weeks and weight of 2807 g were observed. So far, the largest group of children from cryopreserved oocytes has been described in a preliminary study of Borini et al. [58] where 4 cases of developmental anomalies were found (Rubinstein-Taibi syndrome, 47XXX, chromosome 19 hypomethylation, and choanal atresia) in 146 births. Tur Kaspa et al. [59] analyzed more than 37 studies on children from cryopreserved oocytes. In 555 live births, 5 developmental anomalies were identified. This corresponds to a frequency of congenital abnormality comparable to the one that affects spontaneous conceptions. The criteria adopted for this analysis have been questioned. In particular, it has been objected that the overall number of births was overestimated as a result of duplication of sets of original data [60]. However, the absence of an association between oocyte cryopreservation and congenital anomalies is confirmed by a different analysis including over 900 births [61]. Eight children were found affected of various anomalies at birth, a result that, again, is comparable to the frequency occurring in spontaneous births.

The debate on the safety of oocyte cryopreservation remains open. For example, data on the frequency of spontaneous abortion are lacking. Nevertheless, current evidence does not seem to support the existence of specific health risks and legitimate the use of oocyte cryopreservation as a treatment for women requiring an assisted reproduction technology treatment.

53.6 Conclusions

Oocyte cryopreservation by CRSC has become a well-established procedure able to compete with embryo freezing, especially after recent progress in the development of novel protocols and conditions. Safety concerns have not been confirmed, although further and more rigorous studies are needed. Future development of oocyte CRSC will depend on the application of novel theoretical models that can better predict cryopreservation conditions and thereby improve oocyte quality after freezing-thawing.

Review Questions

1. What are the limitations of oocyte slow-cooling approach?
2. Are all the protocols adopted equivalent in term of survival and outcomes?
3. Are data provided from invasive methods valuable to improve SC protocols?
4. Are there any advantages in the laboratory management of female fertility preservation via slow cooling?
5. Is it time to discontinue slow freezing?

Freezing

Freezing Solutions

Freezing Solution 1:	1.5 mol/l PrOH
	1.08 ml PROH
	6.92 ml PBS
	2.00 ml PPS

Freezing Solution 2:	1.5 mol/l PrOH, 0.2 mol/l sucrose
	1.08 ml PROH
	6.92 ml PBS
	2.00 ml PPS
	684 mg sucrose

Notes

(a) Freezing solutions must be stored at 4 °C. Warmed to room temperature (24–25 °C) before use.
(b) All dehydration steps must be performed at room temperature.

Dehydration

(a) For each oocyte (or group of oocytes), dispense 0.5 ml of the freezing solutions in separate wells of a 4-well plate.
(b) Incubate sequentially oocytes in the freezing solutions according to the times indicated below.

Solution	Time
Freezing Solution 1	10 min
Freezing Solution 2	5 min

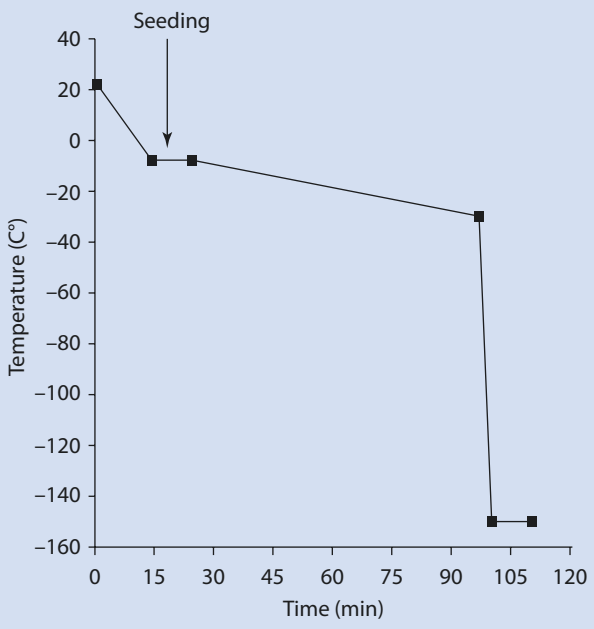
(c) Load oocyte(s) into straw(s)
(d) Seal straw(s)

Cooling to LN_2 temperature

(a) Place straws into the cryofreezer.
(b) Run the controlled rate freezing program (see below).

Controlled rate freezing program

1. Decrease temperature from +20 °C to –7 °C at a rate of −2 °C/min.
2. "Hold" at –7 °C for 10 min.
3. Perform manual seeding at about 30% of the "hold" ramp.
4. Decrease temperature from −7 °C to –30 °C at a rate of −0.3 °C/min.
5. Decrease temperature from −30 °C to −150 °C at a rate of −50 °C/min.
6. Hold at –150 °C for 10 min.
7. Transfer into LN_2 for long-term storage.



Thawing

Thawing Solutions

Thawing Solution 1:	1.0 mol/l PrOH, 0.3 mol/l sucrose
	0.72 ml PROH
	7.28 ml PBS
	2.00 ml PPS
	1026 mg sucrose
Thawing Solution 2:	0.5 mol/l PrOH, 0.3 mol/l sucrose
	0.36 ml PROH
	7.64 ml PBS
	2.00 ml PPS
	1026 mg sucrose
Thawing Solution 3:	0.3 mol/l sucrose
	8.00 ml PBS
	2.00 ml PPS
	1026 mg sucrose
Thawing Solution 4:	
	8.00 ml PBS
	2.00 ml PPS

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Note

(a) Freezing solutions must be stored at 4 °C. Warmed to room temperature (24–25 °C) before use.
(b) All dehydration steps must be performed at room temperature.

Thawing

(a) For each oocyte (or group of oocytes), dispense 0.5 ml of the thawing solutions in separate wells of a 4-well plate.
(b) Remove the straw from LN_2 and keep at room temperature for 30 sec.
(c) Transfer the straw in a + 30 °C water bath for 40 sec.

Rehydration

(d) Release the oocyte(s) from straw(s).
(e) Incubate sequentially the oocyte(s) in the freezing solutions according to the times indicated below.

Solution	Time
Thawing Solution 1	5 min
Thawing Solution 2	5 min
Thawing Solution 3	10 min
Thawing Solution 4	10 min

(f) Transfer the oocyte(s) to 37 °C for 10 min, while in thawing solution 4 and after the initial incubation at room temperature.
(g) Transfer the oocyte(s) in medium for oocyte culture and incubate under standard conditions for 60–90 min before microinjection.

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Vitrification: Methods Contributing to Successful Cryopreservation Outcomes

James J. Stachecki

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Learning Objectives

- To provide a basic understanding of key cryopreservation/vitrification concepts
- To review past research leading to current methodologies
- To discuss concepts important to frozen embryo transfer success

54.1 Fundamentals of Vitrification

The only method of stable and long-term (practically infinite) preservation and storage of any perishable biological materials, particularly cells, is to keep them in the glassy (vitreous) state. This was apparent to Father Luyet when he titled his pioneering work "The *vitrification* of organic colloids and of protoplasm" and "Revival of frog's spermatozoa *vitrified* in liquid air" [1, 2]. He and other *pioneers of the cryobiological frontiers* including Lovelock, Meryman, Mazur, Polge, Smith, Levitt, Farrant, and Willadsen clearly understood some 40–70 years ago that only a glassy state would insure stable and non lethal preservation of cells [3–6]. With time, we saw the development of a variety of biopreservation methods, such as slow-cooling, ultra rapid cooling, and kinetic vitrification [7, 8]. It was Luyet's work that would make cryopreservation a science. From the outset, he recognized that ice damage must be avoided and vitrification could be a method for long-term preservation of cell viability [2].

In order to understand rapid-cooling or "modern" vitrification techniques, let us compare them to the slow-cooling method. During slow-cooling, embryos are exposed to relatively low concentrations of cryoprotectants (1.5 M PrOH and usually some sucrose, around 0.2 M), equilibrated for 10–25 min at room temperature, loaded into a straw or vial, sealed, and placed into a controlled rate freezer. Ice formation is induced extracellularly by seeding at a temperature, whereby ice can perpetuate (around −5.5 °C or lower), and, as a result of the solute gradient created, freezable water flows out of the cells, minimizing the chance of intracellular ice formation during cooling. As the temperature is gradually lowered, the concentration of cryoprotectant in the liquid phase, which includes the intracellular fluid, increases correspondingly until a level is reached at which additional formation and growth of ice crystals, although possible, are unlikely, even if the temperature drops further [9]. Rather, the liquid phase turns into a glassy substance that solidifies without further crystal formation as the temperature continues to decrease. The unfrozen liquid phase remaining within the cells when they are plunged into LN_2 should ideally consist of this glassy substance with all the original cell solutes remaining in solution [9]. This suggests that when we slow-cool cells using a penetrating cryoprotectant such as PrOH, and standard slow-cooling protocols, we are actually vitrifying the cells. Indeed, when we slow-cool human four- to eight-cell embryos, typical survival rates range between 80% and 100% for many IVF centers. These survival rates would not be possible, at least according to Mazur and company, if intracellular ice formation (IIF) were occurring [10]. This correlates well with the theory that slow-cooling is vitrification. Of course, this does not mean that IIF does not or cannot occur, it simply suggests that in conventional embryo freezing protocols, IIF should not be a major source of cell damage. However, if water efflux is inhibited and does not occur in equilibrium, as suggested, and despite a slow-cooling rate, IIF could pose a real problem [11].

IVF laboratories have been cryopreserving spare embryos for more than 30 years. There is no doubt that the technique can work and has great value in the IVF industry. Today the field has changed quite markedly from equilibrium slow-cooling [11] toward a quicker methodology of rapid-cooling. However, from a basic cryobiological standpoint, nothing has really changed. The more recent technique of rapid-cooling is called vitrification by most, despite the fact that slow-cooling was also vitrification. The confusion between slow-cooling and vitrification continues, perpetuated by inconsistencies in the literature.

The basic concept for successful cryopreservation of a cell is relatively simple. Because cells are mostly water, and upon cooling below a certain temperature, water will form ice crystals that can damage the cell, one needs to avoid this by near total dehydration of the cell or by vitrifying the cell. Near total dehydration may work for plant gametes (certain species of pollen and seeds), but has not been successful for mammalian oocytes or embryos. Therefore, the only option would be vitrification. In its most simplest form, the cell needs either (1) to be partially dehydrated, loaded with cryoprotectants (basically alcohols) that can intercalate between water molecules and inhibit crystalline formation upon cooling, and cooled at an appropriate rate to obtain a glassy state inside the cell or (2) simply cooled at an extremely rapid rate to obtain a glassy state inside the cell, without the need for dehydration or cryoprotectants (kinetic vitrification) [7, 8, 12]. Although kinetic vitrification is possible, it is far too costly and cumbersome to be used in IVF clinics today. Thus, dehydration along with the use of permeating cryoprotectants and rapid-cooling by plunging into LN_2 at room temperature is the method of choice today for human oocytes and embryos.

Rall demonstrated that mouse embryos could be vitrified and survive by cooling them in a relatively concentrated solution and cooling at either 2500C/min or 20C/min [13]. This demonstrated (similar to slow-cooling) that vitrification is not rate dependent. However, successful vitrification depends upon one basic concept: cooling and warming rate [14]. Depending on how much cryoprotectant is used (along with the other components of the media; basically salts) will determine what cooling and warming rates will be needed to minimize intracellular ice nucleation and growth.

If we look at the phase diagram (Fig. 54.1.) of a cryoprotectant solution in water, we will find several sections that are either liquid, solid, or a combination of both [14]. In the diagram, Tm = melting temperature; Th = homogeneous ice nucleation temperature; and Tg = glass transition temperature. Only liquid exists in the area above the Tm curve.

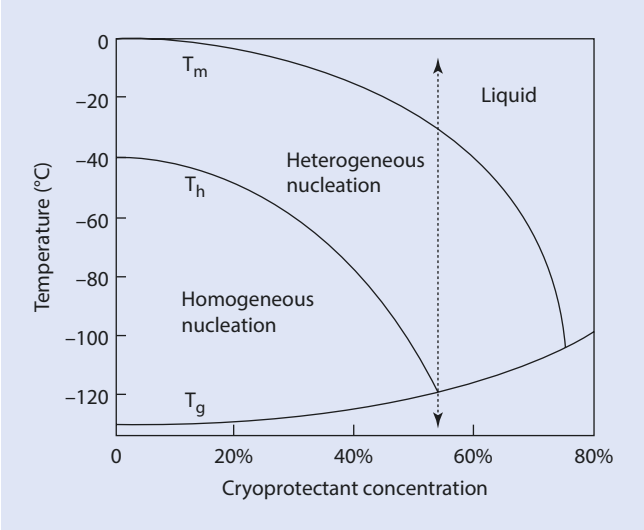

Fig. 54.1 Phase diagram of a cryoprotectant solution in water

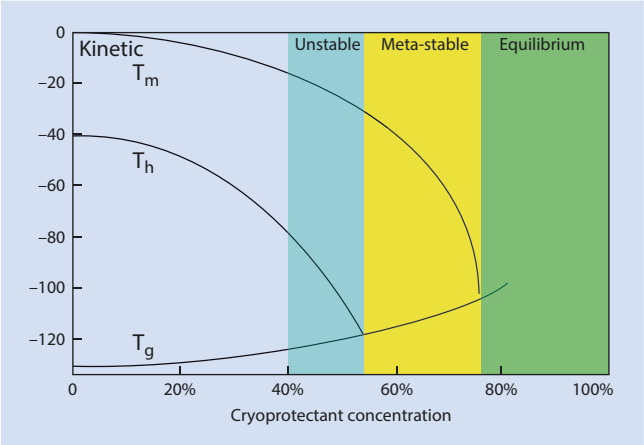

Fig. 54.2 Phase diagram of a cryoprotectant solution in water

Between Tm and Th is the meta-stable region wherein ice can nucleate only by heterogeneous means [14]; basically, nucleation on impurities in the solution (i.e., dirt particles). Below Th, both heterogeneous and homogeneous nucleations exist. Depending on the cryoprotectant concentration, ice can form in a solution upon cooling by either homo- or heterogeneous means. Once the temperature reaches the glass transition temperature Tg, the remaining solution will vitrify. Solidification of water above Tg can lead to intracellular ice formation and cell death.

In Fig. 54.2, we have separated the phase diagram into four sections representing the basic types of vitrification that can occur [14]. On the far left and extending slightly to the right (the clear section), we have kinetic vitrification wherein vitrification can only occur at extremely rapid cooling rates. The blue section, labeled "Unstable," is the area where conventional DMSO-based vitrification solutions lie. Here, we have to worry about both types of ice nucleation and need to cool at a relatively rapid rate (generally >5,000C/min) so that the remaining water in the cells does not nucleate to a great degree and grow during cooling. Also, rewarming has to occur at a rapid rate because the ice that did nucleate during cooling will grow (recrystalize) but can mostly be avoided if rewarming occurs at a fast enough rate. In the "meta-stable" region (yellow) of the phase diagram, we can cool at a relatively slow rate (approximately >20 °C/min) without having to worry about significant ice nucleation upon cooling. Furthermore, as long as rewarming occurs at a relatively rapid rate, we can also avoid recrystalization. The green and final section of the graph represents the area of "Equilibrium" vitrification. Here, one can cool at any rate and not obtain ice formation as the liquid phase transitions directly to the vitrified state upon falling below Tg.

During modern vitrification techniques, we try to avoid ice formation and maximize cell survival [12]. It is clear from looking at the phase diagram that the more cryoprotectant we have loaded into the cell, the smaller the temperature range we need to go through before reaching Tg. At a cryoprotectant concentration of around 20%, one would need to pass from somewhere just below 0 °C to below −120 °C before reaching Tg. If the cryoprotectant concentration was 60%, the range would be approximately −30 °C to −116 °C—a much smaller range. The further right we get in the diagram, the smaller the range between Tm and Tg is and thus the slower the cooling rate and warming rate that is needed to avoid a lethal amount of ice formation. Therefore, it is the cooling and warming rates that are most important and determine the type of storage container needed.

54.1.1 Vitrification Studies: Animal Research

Slow-cooling regimes have been very successful in clinical IVF, simply based on the fact that, conservatively, over 75,000 children have been born from previously frozen embryos. However, there is room for improvement, especially when it comes to storing blastocysts and oocytes. Blastocyst storage using slow-cooling procedures has met with little success for most. Studies by Fehilly et al. [15], Hartshorne et al. [16], Menezo et al. [17], and Kaufman et al. [18] demonstrated that human blastocyst cryostorage was possible; however, in the early 1990s, there was little in the literature regarding this subject. One reason is that IVF clinics, at that time, were just learning how to culture embryos to the blastocyst stage and transferring eight-cell embryos on Day 3 of culture was considered optimal [19]. Therefore, with the relative lack of experience of culturing embryos to the blastocyst stage and the fact that Day 3 storage was relatively good (around 80% survivals), the need did not exist for blastocyst storage in the late 1980s. One common problem among IVF clinics, even today, is that multiple offspring are being produced from the replacement of more than one embryo on Day 3. Multiples dramatically increase the risks and costs of a pregnancy, and thus replacing only a single embryo would be beneficial [20]. The only problem is that pregnancy rates tend to be lower with only one embryo replaced and this is not conducive to

a successful clinic. Replacing blastocysts, however, leads to very good implantation and pregnancy rates, and therefore a clinic could maintain relatively good pregnancy rates and reduce multiples at the same time [19]. As embryo culture progressed and more labs were culturing out to the blastocyst stage, to reduce the number of embryos replaced as well as the number of excess embryos that would have to be stored, blastocyst cryopreservation gained importance.

Much of the work on embryo cryopreservation by the 1990s had been done in domestic species including mouse, cow, sheep, rabbit, and pig, and these studies laid the groundwork for human blastocyst storage [21–31]. One of the first studies done was by Whittingham with mouse blastocysts [21]. Even so, many clinics still struggle with obtaining acceptable success rates by slow-cooling blastocysts [32–34]. By the 1990s, vitrification or, more accurately, rapid and ultrarapid cooling research in animal models was prevalent. The main reason was that several commercially valuable species, including bovine and porcine, were sensitive to chilling injury, and conventional slow-cooling regimes did not work [31, 35–38]. Therefore, the only way to store these cells was to rapidly cool/freeze them from elevated temperatures by direct immersion in LN2, thus avoiding chilling injury.

As vitrification became more popular, more research was done on animals, both for their importance and as model species for humans. A recent PubMed search showed that there were only about 16 papers on animal oocyte/embryo vitrification that were published from 1985 through 1990. There were 46 more the next 5 years up to 1996, and 69 through 1999. After the seminal paper by Vajta et al. in 1998 [31], the field exploded with nearly 200 papers in the next 7 years and 600 from 2006 to 2016. Of course, these numbers are not exact and do not include book chapters, abstracts, and many foreign journals, but they do demonstrate that animal models played a significant role in optimizing vitrification so that it could be used for human work.

54.1.2 Human Research

The work of Vajta et al. with bovine embryos changed history. They demonstrated that DMSO worked very well if used below a certain concentration to eliminate cellular toxicity. This meant that more water would be inside the cell; thus to avoid IIF, a rapid cooling rate was needed, as described in the previous section with a phase diagram. In order to solve the problem of rapid cooling and warming rate, they used very small volumes of media (1−2 ul) in the open end of a tiny straw and direct submersion into liquid nitrogen. Soon everyone jumped on the bandwagon of "vitrification" using DMSO and very rapid cooling rates. The effectiveness of vitrification by this new method in animals led to a flurry of human studies. The new theories soon became prevalent in the literature. Among these the most popular were that high concentrations of cryoprotectants were toxic, and exposure to the final solution with the highest concentration should be reduced to 60 s or less [39–44]; and the faster the cooling rate, the better the survival. From the early 2000s, by the simple adaptation of Vajta's methodology to human oocytes (described in a separate chapter of this book) and embryos, clinics around the globe have successfully stored tens of thousands of cells and produced thousands of "vitrified" babies. Much of the recent research has dealt with optimizing methods and media.

54.1.3 Current Vitrification Technologies: DMSO Methods

Vajta's open-pulled straw paper [31] detailed the formula and methods for successful vitrification of bovine embryos. The basic method is as follows: oocytes or embryos were pre-equilibrated for 10–15 min in 7.5% DMSO and 7.5% ethylene glycol, followed by a short 60 s incubation in 15% DMSO and 15% EG and then immediately loaded into a pulled straw and submerged into liquid nitrogen. Warming was done rapidly by removal of the straw from liquid nitrogen and submersion into 37 °C buffered media containing 1 M sucrose. The cells were then rehydrated by incubation through a series media with reduced sucrose concentration. This methodology has changed little in the past 18 years. The initial success at the turn of the century led to the commercial production of vitrification media by numerous manufacturers, all copying the same basic formula. To differentiate the different companies' products, they all had slight modifications such as using trehalose instead of sucrose, or MOPS buffer rather than HEPES, and using their own base media. One manufacturer even swapped out DMSO for PrOH in the same concentration, which was one of the biggest changes to the original formula, and surprisingly, for some groups, worked well.

If we recall from the previous section, these DMSO-based vitrification solutions lie within the "unstable" or "doubly unstable" region of the phase diagram (Fig. 54.1.), as both types of ice nucleation can occur upon cooling and warming [14]. To avoid this from occurring, or at least minimize it, the cooling and warming rate needs to be relatively high (generally >5,000C/min). In order to achieve this, several new storage devices were developed and tested. The open-pulled straw was good, but manufacturers wanted something of their own they could sell. Soon a variety of plastic straw and spatula devices were developed. Kuwayama et al., [45] measured the cooling rate for a 0.25 cc straw containing 25 ul of vitrification solution plunged directly into liquid nitrogen at 4460 °C/min; an open-pulled straw containing 1.5 ul of vitrification solution at 16,340 °C/min and a cryotop with 0.1 ul of vitrification solution at 22,800 °C/min. Similar products including electron microscope grids, cryo-top, cryo-tip,

cryo-leaf, and nylon loops that all allowed direct contact with liquid nitrogen and micro volumes were able to increase the vitrification speed considerably, in the order of >15,000 °C/min. Clinics testing these new devices soon reported high survival rates [36, 41, 43, 45–54]. Some of these devices including micro-secure, CBS high-security vitrification straw, rapid-i, and cryo-pette are closed so that the cells do not contact liquid nitrogen, thus avoiding any potential contaminants [55–58]. Although the cooling rates for closed systems tend to be slower than open systems, they still work well and achieve rates over 5000 °C/min. Today there are over 20 different micro-volume devices available.

Although the DMSO/EG method has been widely accepted and is used worldwide, the major drawbacks are the potential toxicity of DMSO, short equilibration time, large learning curve to perfect the method, and cost of the tiny plastic storage devices. DMSO is a very good vitrificant; however, it is one of the most toxic [59, 60]. Therefore, exposure of oocytes or embryos to concentration above 6 M is not recommended. More concentrated solutions, above 6 M, are toxic and lead to cell death. Exposure times for the 7.5% solutions range from 5 to 15 min. Because the cryoprotectant concentration is half of the working vitrification solution, the longer exposure time has no overt negative effect. However, when the full strength vitrification solution of 15% DMSO and 15% EG is used, the exposure time must be reduced to around 30–90 s. Longer times that would allow better equilibration (more dehydration and increased loading of cryoprotectants) prove toxic. The end result is that the cells are not completely equilibrized with the cryoprotectants and thus have more water inside. The intracellular water/cryoprotectant concentration places the cells in the "Unstable" range of the above phase diagram (see Fig. 54.2.) [14]. The technical skill in getting the timing correct and loading the cells onto/into the storage device in the allotted time period has led to variable results [61]. Furthermore, the reduced time in full strength vitrification solution is not enough to allow the cryoprotectants to reach the inner cell mass cells (ICM) and protect them. Because blastocysts are morphologically very different from a non-cavitating cleavage-stage embryo, their storage has presented different challenges. The main problem is that the blastocoel is made up mainly of water that can form ice crystals when the temperature is lowered and thus cause damage to the ICM and trophectoderm. To overcome this problem, some investigators have tried collapsing the blastocoel either by pipetting the blastocyst in and out of a fine bore pipette or by rupturing it using an intracytoplasmic sperm injection (ICSI) needle or similar device [48, 51, 62]. Although these papers report increased survival rates using these methods, the obvious drawback is that an additional procedural step is involved that is potentially damaging to the embryo. The collapse is necessary with expanding blastocysts over 150 um in size [63]. The rupture of the trophectoderm allows cryoprotectants rapid access to the ICM, allowing them to be successfully vitrified. In conclusion, DMSO systems work well despite the above-mentioned issues. Not every lab has achieved great results, and variability in success rates within and between labs still exists.

54.1.4 Non-DMSO Methods

Despite the variety of different companies that offer a vitrification system, they are all basically the same. However, not every vitrification system uses DMSO. There is one other method that is vastly different, yet very successful for vitrification of eggs and embryos. Shortly after the open-pulled straw paper came out, Stachecki developed a very different vitrification system and later published on the S^3 method [64, 65]. It was based on the fact that slow-cooling is also vitrification and that this could be achieved in a relatively large container, namely, a 0.25 cc straw. The system was developed around the simple, inexpensive, closed, sterile cryo-straw as the storage container of choice. To avoid the shortcomings of the Vajta method, no DMSO was used, thus avoiding the cryoprotectant toxicity problem. Higher concentrations of cryoprotectants were used without cell toxicity [65, 66]. Combined with longer exposure times in the final vitrification solution, the cells are better prepared for vitrification. A higher concentration of cryoprotectant and longer exposure times result in less water in the cells and a meta-stable environment, as shown in the phase diagram of Fig. 54.2. All combined, a slower cooling and warming rate is needed, allowing for larger containers and more volume to be used, while still achieving the required rates for successful vitrification. The protocol is very forgiving allowing leeway in almost every step, and almost no learning curve is required. The overall results are similar to results using the DMSO system [66].

54.1.5 Vitrification Outcomes

The efficacy of vitrification is ultimately measured by live births. There are many variables that can affect the reported outcome, such as embryo quality, day of vitrification, blastocoel collapse, and biopsy to name a few. Collectively, the results are very supportive of blastocyst vitrification. Clinical outcomes from DMSO vitrified blastocysts have been widely published. Survival and clinical pregnancy rates range upward of 99% and >65%, respectively, depending on the clinic [49, 51, 67–75]. In 2015, Sparks showed more recent blastocyst data with similar high rates [76]. Over the past 5 years, there have been numerous abstracts with reported outcomes from non-DMSO methods, namely, ICE vitrification, that were presented at three major reproductive meetings (ASRM,

Table 54.1 Clinical pregnancy rates of ICE vitrified blastocysts vs. fresh blastocysts

Age	Cryo preg. rate	Fresh preg. rate
<38	771/1300 (59.3%)	263/477 (55.1%)
38–43+	241/419 (57.5%)	42/126 (33.3%)
Donor	138/216 (63.9%)	102/178 (57.3%)

Clinical pregnancy rates (number of pregnancies/transfer) of blastocysts cryopreserved with the ICE vitrification system vs. fresh non-vitrified blastocysts. Data gathered from nine clinics between 2011 and 2016. Blastocyst survival was >93%

PCRS, and AAB) but few publications [65, 66]. We report here (Table 54.1) a collection of clinical results from nine different clinics over a 5-year span from 2011 to 2015 using the ICE vitrification system. Collectively, survival rates were >93%, and the overall clinical pregnancy rate was 59.4% in 1935 transfer cycles.

54.1.6 Emerging Concepts

During the past 15 years, vitrification has been adopted by almost every clinic in the USA and most others worldwide. With the ability of blastocyst culture and genetic testing at the blastocyst stage, vitrification of embryos as well as oocytes has revolutionized the IVF industry. The DMSO and S^3 systems were so highly successful and much better than anything else available at the time. Not surprisingly, the high rate of success has stifled innovation and further development of cryopreservation systems. Companies trying to separate their system from others have used various buffer systems, or different sugars (sucrose vs. trehalose, a disaccharide present in certain species that are able to remain vitrified for years), or even swapped PrOH for DMSO, but in the end, they are all, cryobiologically speaking, similar.

There are, however, several emerging concepts in the field that are worth mentioning. Kuwayama's split with the Kitazato Corporation led him to rethink his DMSO system, which resulted in CryoTec™, a modification of the basic DMSO system but with hydroxypropylcellulose (HPC) rather than human serum albumin (HSA) as a protein source. HPC, like albumin, is a plasma volume expander, effectively reducing water (available to form ice crystals) and increasing solution viscosity. HPC, unlike HSA, is a fully synthetic macromolecule, thus avoiding endotoxin and other possible contaminants. Coello et al. [77] and Mori et al. [78] recently showed that HPC and trehalose were safe substitutes for HSA and sucrose for vitrification of oocytes or embryos and that there was no difference between survival rates, embryo development, and pregnancy rates.

To further develop and perfect the S^3 vitrification system, Stachecki optimized protocols to reduce the time and still allow leeway in the protocol. He also introduced hyaluronate into the media (unpublished results). Hyaluronate is known for its beneficial embryo growth and development properties, as well as its ability to increase viscosity [79–84]. The new and improved system was called I.C.E. vitrification and represented a significant improvement in an already viable system [66].

Katkov has worked on developing a purely kinetic system for vitrifying sperm without the use of cryoprotectants [8]. Although he has showed feasibility, more work needs to be done if it is to enter in the human IVF field.

Recently, there have been several automated vitrification systems in the work: Gavi™ from Serono and a system from ESCO. These machines are large, complex, and, at times, not so automated. Clinical testing and continued development of these prototypes may lead to a more simplified and time-saving approach.

54.2 Beyond Vitrification: Other Factors Affecting Outcomes

54.2.1 Embryo Culture

Vitrification of human gametes and embryos is extremely successful. Published success rates and overall clinical success has changed the field and how we do IVF. This has come about due to a number of reasons; first and foremost is the improvement in culture conditions. Embryo culture to Day 5 and beyond has improved significantly in the past 15–20 years, and blastocyst transfers are now the norm, at least in the USA. Second, it has been assumed that the uterine environment, in a controlled ovarian stimulation (COS) cycle, may not be as good as in a natural cycle or with controlled preparation for a frozen embryo transfer cycle [85, 86]. If this is true (and the published evidence is rather convincing [87–94]), freeze-all cycles will avoid the transfer of fresh embryos to a stimulated uterine environment that can be suboptimal for implantation and development. The end result is that more and more clinics are using both single embryo transfer (SET) and freeze-all cycles, especially for their older (>37y.o.) patients [95]. SET has been shown to reduce multiple pregnancy rates, and the risks and costs associated with twins and multiples still maintain a high take-home baby rate [86]. Collectively, the improvement in embryo culture, improved genetic testing and blastocyst biopsy, and avoidance of transfer in a fresh cycle have perpetuated the need for the safe and efficient storage of blastocysts.

There are a number of factors that affect the overall success of vitrification, yet have nothing to do with the actual vitrification or warming process. These start with patient demographics, the stimulation protocol, culture conditions, and anything else that is associated with affecting the overall embryo quality. No matter how good the vitrification system is, poor embryo quality, either inherent or caused by user error and/or poor culture conditions will not, at least most of the time, lead to success. Because of the myriad of other factors that can influence outcomes, troubleshooting a

vitrification system is challenging. The whole system must be accounted for from egg collection through warming, culture, and transfer. Despite these issues, many clinics are obtaining better overall pregnancy rates with vitrified embryos than fresh ones.

54.2.2 Patient Preparation and Embryo Transfer

Unfortunately, many good embryos fail to produce a baby, even with good embryo culture and a good vitrification system [83, 96] [97–100]. A final piece in the overall puzzle of producing a healthy pregnancy, or at least a successful positive pregnancy with a fetal heart beat, is the embryo transfer procedure [83]. The transfer procedure can be affected by numerous factors including patient-specific issues, contamination of the catheter, retained or expelled embryos, type of catheter, media used, adjuvants, timing, etc. [83]. It is obviously important to perform the transfer correctly, but not so obvious is the proper uterine preparation [99], which has been the cause of many failed cycles. As the number of clinics increase their FET rates and/or move to freeze-all cycles, one would assume that, based upon the evidence in the literature, the pregnancy and take-home baby rate would naturally increase. However, improper uterine preparation leads to an increase in both failed and biochemical pregnancies. Because suboptimal preparation is difficult to determine, it often goes unnoticed for months. Analyzing biochemical, clinical pregnancy, and delivery rates over time and between cycles will show that something is amiss. An overall increase in biochemical pregnancies and decrease in fetal heart beats and/or delivery rates over a period of time can be a strong indicator of poor uterine preparation.

A pregnancy will initiate only when the embryo is ready to implant and only when the uterus is ready for implantation. This window of implantation, although typically 12−24 h wide, varies and can be different between patients [101]. Proper uterine preparation is key to synchronizing the implantation windows of the uterus and embryo [96, 99]. Casper and Yanushpolsky recently reviewed this topic in 2016, and their work can serve as a good source for additional information as this chapter will only briefly touch on this topic.

Endometrial morphology is an appropriate predictor of receptivity for implantation, and hormonal control of endometrial receptivity includes an estrogen priming phase followed by progesterone (P4) secretion, which leads to the necessary endometrial changes [102, 103]. Estrogen increases endometrial thickness and is given for approximately 2 weeks until thickness reaches around 7 mm. At this time, P4 administration begins for the number of days proportional to the embryo stage [104]. Thus, a Day 5 blastocyst would require approximately 5 days of P4. However, it is known that approximately 25% of women can be out of phase and would require longer P4 administration prior to embryo transfer [105, 106]. The dosage and timing is very important. For instance, it is widely suggested to do the thaw and transfer of a D5 blastocyst on D6 of P4 IM administration [105, 107]. Alternatively, we do not want to advance the endometrium using too much P4 and close the window of implantation too early. Despite the individual differences between patients, which may lead to failed pregnancies, the basic idea is to optimize the uterine preparation so that overall FET pregnancy rates are high and biochemical pregnancies remain at or below fresh ET levels.

The type of progesterone is very important and can make a difference in pregnancy rates [108–110]. However, randomized controlled studies have shown both vaginal and intramuscular P4 to be equally effectively [111–115] with an overall similarity in pregnancy rate. There are pros and cons with both vaginal and IM P4, and these should be considered prior to use. The short half-life of natural P4 (used in vaginal supplements) dictates multiple daily usages in order to maintain natural P4 serum levels. By contrast, IM P4 in oil has a continuous release over time and thus a longer half-life, requiring less administration [110, 116]. However, because of its route, it takes much longer for IM P4 to start affecting the uterus, which can lead to a longer administration period prior to ET [110].

Progesterone can also affect uterine contractility and thus influence implantation and pregnancy rates [117]. It has been shown that increased myometrial contractions are associated with decreased pregnancy rates [118, 119] and tubal ectopic pregnancies. Fanchin showed that the rate was correlated to serum P4 levels; with higher levels correlating to lower contractility and vice versa [120], therefore, performing embryo transfer during a time of low uterine contractility results in better pregnancy outcomes. Estrogen increases uterine contractility and subendometrial wave action, whereas P4 antagonizes this action. Cicinelli showed that endometrial concentrations are higher with vaginal P4 versus IM P4 [116], suggesting that the higher serum levels of P4 after IM administration may work better to reduce uterine contractions. This may be a reason that many physicians still use IM P4, despite the sometimes painful injection regimen. The gap between administration of vaginal P4 and the actual time of FET the following day can be large enough to result in a low P4 concentration in the uterus and greater contractions. This gap does not occur with IM P4, due to the continued release and higher serum concentrations. Casper therefore suggests that IM P4 may quieten endometrial activity better than vaginal P4, at least until implantation where a switch to vaginal P4 administration could be considered [110].

54.3 Conclusion

There are many factors that can affect the outcome of a vitrification procedure, from embryo quality, patient demographics, culture conditions, and laboratory quality, to the actual vitrification and warming procedure, uterine preparation, and the embryo transfer itself. All of these variables must be carefully orchestrated and managed in order to

achieve a successful outcome. Embryo and gamete storage has come a long way from the discovery of glycerol in Polge's lab to the successful cryopreservation of mouse embryos over 40 years ago to modern ultrarapid cooling techniques. From the start, animal models have provided us with most of the breakthroughs in the area of cryobiology and embryo storage. It is these pioneering studies, along with new innovative ideas, that hold the key to even more breakthroughs. Emerging methods of kinetic vitrification, as well as more robust meta-stable solutions, and simple, safe, and effective storage containers like the micro-secure will lead us into the future [7, 66, 121]. It can all be seen as one big, extensive research project culminating in the ability to successfully preserve a wide variety of mammalian embryos in a rather simplistic manner with minimal resources, namely, a set of cryoprotective solutions, a storage device, and a bucket of liquid nitrogen. Despite its simplicity, we must keep in mind that these cells were not meant to be frozen, and that any success at all is truly remarkable. Although we have seemingly conquered, or at least been very successful at storing human embryos and gametes, there is a lot we do not understand about the actual vitrification and warming process. Not all cells survive storage, and those that do, not every embryo successfully implants and continues to develop. When cells do not survive we assume it is from intracellular ice formation or osmotic effects or toxicity, but we really are just guessing [11]. We have no way of seeing what is happening inside the cell and can only make assumptions, many of which are incorrect. These unknowns and the continued push to find simpler, more effective methods of embryo storage keep us looking to the future for a deeper understanding of the complexities of human embryo vitrification.

Review Questions

1. What is the key factor(s) for vitrifcation of a cell?
2. How is slow-cooling the same as vitrification?
3. What are the two basic types of vitrification systems available today and how do they differ?
4. Besides performing basic tasks appropriately (i.e., vitrifying correctly or performing the embryo transfer correctly), what are the three key factors for a successful FET outcome?

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Oocyte Vitrification and Current Clinical Applications

Ana Cobo

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Learning Objectives

- To understand the basic principles of vitrification and how we can achieve it in our clinical settings
- To analyze the contribution of oocyte vitrification to clinical practice
- To describe the methodology, logistics, and technical aspects related to oocyte vitrification

55.1 Introduction

The essential role of cryopreservation in ART has become obvious since the establishment of the infertility treatment, bringing flexibility and efficiency to the practice. Semen and embryo cryopreservation has been a successful strategy, and its routine application is a reality since the onset of the IVF practice. However, in spite of numerous studies conducted over two decades, the reliability of oocyte cryopreservation is relatively recent. All of the efforts made are clearly justified, mostly because an efficient oocyte cryopreservation program is actually quite welcome and widely applied in ART, since there are many indications for this strategy, including egg-banking for ovum donation. Beneficiaries of this approach include cancer patients who need an option for fertility preservation before undergoing their oncological treatment [1], or women who wish to delay their motherhood due to a variety of reasons [2], government restrictions on IVF [3, 4], ethical reasons against embryo cryopreservation and practical reasons such us unavailability of the male gamete the day of pickup [5, 6].

Despite all of this wide diversity of potential applications, egg-banking has not been a routine procedure until relatively recent. This fact can be explained because the methodology to cryopreserve human oocytes was disappointing, with results that have not always been reproducible, especially during the 1980s and 1990s. During the early 2000s, vitrification brought efficient and reproducible outcomes to the clinical practice. Some fundamental principles of cryobiology would be helpful to understand how difficult it has been to reach the goal of safe cryopreservation of human oocytes.

55.2 Cryobiology Background

There are some reasons that could explain the low rate of successes initially observed with the first protocols available for oocytes cryopreservation. At first, the size and shape of the oocytes are intrinsic cell features that strongly challenge the outcome. These gametes are the largest cells of the human body, fact that could explain, at least in part, the great differences in cryotolerance between them and, for example, the spermatozoa. In addition, the spherical shape of the oocyte could disturb the uniform distribution of cryoprotectants (CPAs) along the cytoplasm. In addition, considering the oocytes as a unique cell, there is 50% choice of survival and 50% choice of cell death. This situation is completely different from the one observed in tissues composed of millions or thousands of cells, i.e., semen samples, in which the damage could be compensated in different proportions.

Other major factors responsible for the high oocyte sensitivity to cryopreservation include chilling injury and intracellular ice crystal formation. These factors are related to the cryopreservation method. There are two main strategies in cryobiology: slow freezing and vitrification. During the former, the cells are gradually dehydrated in the presence of CPAs, and the temperature is lowered at a very slow cooling rate (−0.3 °C) [7]. In this way, the cells are exposed to low temperatures during an extended period of time, which can lead to what is known as chilling injury [8]. At the final stage, water solidifies into ice crystals. Chilling injury can be defined as the irreversible damage following exposure of cells to low temperatures, before the nucleation of ice [9]. This detrimental event affects mainly the cytoskeleton [10] and cell membranes [11]. The ice crystal formation within the cytoplasm must be avoided at all cost in order to guarantee the survival and integrity of the cells when they are later thawed. Chilling injury can be minimized during vitrification by the use of high cooling rates [12].

Vitrification, in particular by means of methodologies that use a minimum volume, yields extremely successful outcomes, that have not been achieved with other approaches [6]. The physical phenomenon of vitrification takes place when the solidification of the solution occurs not by ice crystallization but by extreme elevation in viscosity, which is achieved by using extremely high cooling rates from −15,000 to −30,000 °C per minute, allowing to avoid the risk of chilling injury [12]. This ice-free cryopreservation method has been modified in order to optimize results. One such modification has been to reduce the volume of the vitrification solution containing the oocytes, which allows the CPA concentration and, consequently, the cytotoxicity to be decreased [13, 14]. As mentioned, this procedure circumvents the two major limiting factors for achieving optimal cryopreservation: chilling injury [14] and ice formation [15]. Chilling injury can be minimized during vitrification by the use of high cooling rates in order to avoid the range of temperature at which chilling takes place (+15 °C to −5 °C) [12]. Additionally, the velocity of the process is dependent on the volume of the vitrification solution. Thus, the smaller the volume of the sample, the higher the cooling rate. On the other hand, direct contact with liquid nitrogen also contributes to increase the cooling rate. Ice formation can be also avoided during vitrification by the use of high CPA concentrations [12], despite the fact that such high concentrations are considered toxic to cells [16]. Nonetheless, an appropriate, phased composition of CPA could mitigate the toxic and osmotic consequences of highly concentrated CPA mixtures [6]. In this way, a combination of two or three of these agents can decrease the individual specific toxicity of these agents. The most common mixture employed for this purpose consists of ethylene glycol, dimethyl sulfoxide, and sucrose or trehalose [6]. To optimize the results, in addition to an appropriate selection of CPAs, it is also helpful to use these agents

at as low a concentration as possible, while maintaining the necessary composition able to achieve vitrification. By dramatically increasing the cooling rate, the CPA concentration could be reduced. As a result, a high cooling rate avoids chilling injury, and allows the reduction of the concentration of CPA, thereby preserving the cells at nontoxic concentrations of cryoprotectant. Several approaches fit these conditions. The "minimum drop vitrification" method, proposed by Arav, uses a very small volume of vitrification solution by placing the samples on a specific device that must be cooled very quickly [17]. Extremely high cooling rates are achieved when samples loaded in minimum volume are directly immersed in LN. These methods are also known as open systems. Hermetically closed vials achieve lower cooling rates as compared with open devices. Nevertheless, it is worth mentioning that the direct contact of samples with LN has raised some concerns due to the theoretical risk of cross-contamination mainly because the LN itself is considered as a potential source of pathogen agents, even though no case of cross-contamination has ever been reported in ART in many years of practice, highlighting that such eventuality could be extremely unlikely.

A wide variety of open approaches have been reported in the literature [18–23] as well as closed ones [24, 25].

55.3 Contribution of Oocyte Vitrification to Clinical Practice

55.3.1 Ovum Donation Programs

Oocyte cryo-storage results are very useful to overcome the most common drawbacks involved with ovum donation as currently applied, such as synchronization between donors and recipients, long waiting lists subject to the availability of a suitable donor, and most importantly the absence of a quarantine period.

55.3.1.1 Management of Egg-Banking in our Clinical Setting

Egg Donor Selection

Spanish Assisted Reproduction Law is based on legislation that was passed in November 1988 (Law 35/1988) (► https://www.boe.es/buscar/doc.php?id=BOE-A-1988-27108). Although some countries already had regulations on, Spain was the first country to create a specific law to cover this area of medicine. Royal Decree 412/1996 and Ministerial Order of 25 March 1996 establish donor requirements, as well as mandatory standard screening procedures, to rule out the transmission of genetic, hereditary, or infectious diseases (► https://www.boe.es/buscar/doc.php?id=BOE-A-1996-9723). In 2006, the newly approved Spanish Law on Assisted Reproduction (Law 14/2006) (► https://www.boe.es/buscar/act.php?id=BOE-A-2006-9292) regulates the requirements for gamete and embryo use and rules on financial compensation aspects.

These are the most important topics included in Spanish Law on Egg Donation:

- *Donation of human gametes is a formal confidential contract between the donor and the reproductive medicine center. Identity of donors must remain anonymous.*
- *The donation cannot be revoked.*
- *The maximum number of children generated from a single donor's gametes should not exceed six.*

To be accepted as an egg donor, women must be aged between 18 and 35 years old and be healthy. The following steps are necessary for the admission as an egg donor in our clinics:

- *Medical History*: During the first consultation, the applicant is interviewed in order to complete the familiar and personal history.
- *Psychological Screening*: Psychological evaluation and counseling by a qualified mental health professional. The potential donor is evaluated by a psychologist, to ensure that she fully understands the benefits and risks of egg donation, and is properly motivated to become a donor.
- *Gynecological examination*: Menstrual cycles, ovaries examination by ultrasound and antral follicles count are performed. At the same time, body mass index is calculated.
- *Medical Screening*: This involves testing for blood type, antibody screening, complete blood count, hemostasis, biochemistry and infectious disease screening, such as HIV, CHV, CHV, and syphilis among others.
- *Genetic Screening*: Blood tests for karyotype and for carrier screening tests for severe recessive and X-linked childhood diseases based on NGS (549 genes implicated in 623 disease phenotypes).

Ovarian Stimulation

To initiate, an oral contraceptive pill is administered for a maximum of 21 days, which starts on day 1 or 2 of the menses of the previous cycle [26]. After a 5-day washout period after taking the last pill, donors start their stimulation protocol with 150–225 IU of recombinant FSH, 225 IU of HP-hMG, or 150–225 IU of recombinant FSH plus 75 IU HP-hMG. Regular vaginal ultrasound monitoring of donors is performed during FSH injections to measure follicle growth. Daily doses of 0.25 mg GnRH antagonist (Ganirelix or Cetrorelix) start on day 5 of stimulation. Once the leading follicle reaches 18 mm in diameter, a single dose of GnRH agonist is administered to trigger the final oocyte maturation. Transvaginal oocyte retrieval takes place 36 h later after GnRH agonist administration. Donors receive light sedation for the egg retrieval procedure to ensure their comfort, and they rest for 2 h at the clinical setting until they are discharged. In some cases, a vaginal ultrasonography scan is scheduled 2–3 days following egg retrieval [27].

55.3.1.2 Oocyte Recipients

Oocyte recipients enter our egg donation program for one of the following main diagnoses: premature ovarian failure/menopause, failure to achieve pregnancy after at least three

cycles of assisted reproduction techniques, genetic or chromosomal disorders, low response to controlled ovarian hyperstimulation, and recurrent miscarriages.

The vast majority of oocyte recipients undergoes hormone replacement therapy (HRT). In patients with ovarian function, depot GnRH-a is administered in the midluteal phase of their cycle, or GnRH-ant, administered daily with menstruation for 5 days. HRT is initiated on days 1–3 of the following cycle with oral estradiol valerate or an estradiol transdermal patch [28–30]. Recipients without ovarian function are submitted to the same endometrial preparation protocol but are not administered depot GnRH-a. On day 15 or 16 day of HRT, a transvaginal ultrasound is performed to measure endometrial thickness, and serum E2 and progesterone levels are tested. Most recipients are ready to receive embryos within 2–3 weeks after starting HRT, although administration of estradiol valerate can be maintained for a maximum of 50 days until a suitable donation becomes available. Micronized progesterone (800 mg/d, vaginally) is initiated on the day after oocyte donation, and embryos are transferred in the blastocyst stage.

The recipient continues taking estrogen and progesterone with a positive pregnancy test, and these hormonal supplements are then continued through 12 weeks of pregnancy.

Before treatment begins, the recipient undergoes preliminary testing. This assessment phase includes infectious disease screening, e.g., HIV, CHV, CHV, syphilis, and blood type, and Rh factor for both parents. In women older than 45 years old, a recent mammogram, full blood count, coagulation tests, and blood biochemistry may also be required.

55.3.1.3 Donor-Recipient Matching Process

In our center, we have developed a consistent software tool that accounts for different characteristics of the woman and her partner, as phenotypic features, blood type, etc. The tool provides a list of available donors and number of oocytes available, thus simplifying greatly the process of donor-recipient matching.

Matching is known as the time when we select a donor for a recipient after taking into account several features. We consider many different factors during donor selection: race, reproductive history, the physical characteristics that match those of the female partner, blood type, and genetic carrier screening.

The time for donors-recipients matching has changed in the last 6 years thanks to the improvement in the oocytes vitrification technique and the establishment of the egg donor bank. However, it is important to note that in our current practice, we conduct donations both with fresh and vitrified oocytes, as long as fresh donations are still allowed in our country. The decision about conducting one strategy or the other depends on different circumstances related to the availability of oocytes and the needs of the recipient.

If there is the case that the recipient requires particular rare characteristics, i.e., blood type (0 negative, AB negative), specific race, screening for specific genetic diseases, or partners who would like to have another baby with the same donor as they had before, the procedure would be as follows:

- First, we use our donor selection database and select one donor or two with the required characteristics. If there are no suitable donors undergoing ovarian stimulation by the moment of the matching, we search for another appropriate donor within the list provided by the tool after the matching process and ask them for their availability to return to initiate the ovarian stimulation for this specific donation.
- Second, all the oocytes obtained during pickup are vitrified and reserved for the recipient.
- Finally, the recipient chooses the best time to schedule embryo transfer, and we provide them with instructions to begin HRT depending on the day of the embryo transfer.
- Recipients who do not need specific characteristics:
  - The date for embryo transfer is already set.
    - First, we reserve oocytes from our egg donor bank.
    - Second, the recipient begins HRT depending on embryo transfer.
    - Finally, we have two options:
  - We use fresh oocytes when we have a donor pickup scheduled on the same date as the donation (with the same characteristics as the partner). The prior reservation of oocytes is canceled in these cases.
  - The date for embryo transfer is not set yet.
    - The recipient starts HRT and remains on the waiting list
    - If on these dates an egg donor with the same characteristics as the recipient undergoes pickup, we use fresh oocytes for the egg donation.
    - If the recipient stays on the waiting list longer than 20–25 days, we use oocytes from the egg donor bank.

55.3.1.4 Clinical Outcome

The first live birth after vitrification was achieved from a donated oocyte using an open system device (OPS) in 1999 (Kuleshova et al. [33]). Since then, numerous publications using different types of devices corroborate the effectiveness of the technique. ◘ Table 55.1 shows a list of the survival and pregnancy rates by different groups using donated vitrified oocytes.

In 2008, our group published a study aimed to assess the impact of vitrification using donated oocytes, in terms of survival and development potential when compared to fresh oocytes (Cobo et al. [1, 5]). A cohort of oocytes from a single donor was randomly divided into two groups: in the first one, the oocytes were vitrified, while in the second one, the remaining oocytes were kept in the incubator (control fresh oocytes). Oocytes were warmed up 1 h after vitrification. After two hours, we proceeded to simultaneous insemination of the fresh and vitrified oocytes. The Cryotop method employed for oocyte vitrification was that previously described by Kuwayama (22). The study included 30 donors and 30 recipients (509 oocytes; 231 vitrified oocytes and 219 fresh oocytes). After a survival rate of 96.9%, we found no differences in the rate of division in day 3 (77.6% vs. 84.6% in vitrified in fresh) or the morphological quality of embryos (80.8 vs. 80.5% of good quality embryos vitrified vs. fresh).

Table 55.1 Key publications related to donated vitrified oocytes

	Year	Device	Survival rate (%)	Pregnancy rate (%)
Kuleshova et al.	1999	OPS	64.7	33.3
Lucena et al.	2006	Cryotop	89.2	56.5
Cobo et al.	2008	Cryotop	96.9	65.2
Chang et al.	2008	Cryotop	85.4	83.3
Sher G et al.	2008	Cryoloop	96.1	81.2
Nagy et al.	2009	Cryotop	89.0	75.0
Cao et al.	2009	Cryoleaf	91.8	
Cobo et al.	2010	Cryotop	92.5	55.4
Noyes et al.	2010	Cryotip	87.0	63.0
Trokoudes et al.	2011	Cryotop	91.4	55.6
García et al.	2011	Cryolock	89.4	61.8
Stoop et al.	2012	CBS high security	90.2	50.0
Papatheodorou et al.	2013	Vitrisafe	90.9	28.0
Solé et al.	2013	Cryotop	85.6	53.5
Figueira et al.	2014	Cryotop	77.1	37.7

Likewise, both the blastocyst rate (48.7% vs. 47.5%) and quality of embryos (81.1% vs. 70%) was similar comparing fresh and vitrified.

Another randomized, prospective, triple-blind, controlled-clinical trial, which included the largest sample size published to date, aimed to validate ovum donation via egg-banking [31]. The study included 600 donor/recipients (300 per each branch) and 3039 vitrified versus 3185 fresh oocytes. In this study we demonstrated the non-inferiority of vitrified oocytes regarding ongoing pregnancy rate by intention to treat (Odds ratio = 0.921, 95% CI 0.667 to 1.274). The overall survival rate in this study was 92.5% and ongoing pregnancy rate by intention to treat was 43.7% in the case of patients who received vitrified oocytes compared to 41.7% obtained with fresh oocytes. This study definitively confirmed our previous observations about the non-alteration of vitrified oocytes potential to develop into embryos capable of generating competent ongoing pregnancies in a similar proportion to fresh oocytes.

The availability of surplus embryos to cryopreserve after the embryo transfer in ovum donation cycles conducted with vitrified oocytes is quite common due to the high developmental potential of these oocytes. In these cases, two rounds of vitrification are involved, at MII stage and at day 3 or blastocyst stage. A study published in 2013 showed that the double vitrification has no effect on the delivery rate or live birth rates (Cobo et al. 2013). This study included 796 vitrified embryos generated from vitrified oocytes ($N = 471$ cycles) and 4394 vitrified embryos generated from fresh oocytes ($N = 2629$ cycles). The overall embryo survival rate was 97.2% (95% CI 95.9 to 98.6) versus 95.7% (95% CI 94.9 to 96.4) for double versus once vitrified embryos, respectively (NS). The live birth rate per warming cycle was 33.8% versus 30.6% (NS). As showed, the odds ratio of the delivery rate and the double vitrification had no effect on embryos regardless of whether they were in early stage development (day 3) or blastocyst stage (OR = 0.867; 95% CI 0.657 to 1.203).

A couple of years ago, we published an observational study in which our experience of 6 years with the egg-banking program (Cobo et al. [34]) was described. The analysis included 3610 cycles of oocyte donation where 42,152 MII were warmed. Survival rate was 92.6%. The impact of storage time on the survival rate and clinical outcome was calculated by establishing different time intervals from less than 6 months to over 5 years. The storage time did not impair survival or clinical outcomes in any case. We believe that this is a very reassuring information, especially for those women considering fertility preservation. The clinical, ongoing pregnancy, and delivery rates were 55.0%, 45.3%, and 37.6%, respectively, thus confirming the consistency of the results as compared to our previous findings (Cobo et al. [1, 5, 31]). The likelihood of having surplus embryos available for additional cryotransfers was very high in this series due to the mean number of oocytes donated. The possibility of further cryotransfers increased cumulative outcomes, and thus maximized the yield of a single donation cycle, which is precisely what we show herein. The cumulative delivery rate per donation cycle increased to over 70% after three cryotransfers and rose to nearly 80% after five cryotransfers. These results render the donation cycle as highly efficient.

Kaplan-Meier analysis observed in Fig. 55.1 provides interesting information about the number of vitrified oocytes consumed by a recipient (either in one or more cycles of ovum donation) necessary for a newborn. The analysis showed that the live birth rate increases exponentially and the patient can achieve a baby with a probability of nearly 100% when about three to four cycles of egg donation (around 35–40 oocytes) are completed. This result confirms our previous findings while showing the excellent efficiency of egg-banking.

55.3.2 Fertility Preservation

Vitrification of oocytes is currently being offered as an option for women who wish to preserve their gametes to allow them to have a chance to conceive in the future and to have their own genetic offspring (Cobo et al. [34]). The main beneficiaries of this strategy are cancer patients who must undergo chemotherapy or radiotherapy and patients with other diseases who require potentially gonadotoxic treatments.

The first reported case in Europe of a pregnancy after FP using vitrification of oocytes was from our group in a patient whose ovarian cortex was cryopreserved first, and after grafting, four stimulation cycles were performed to accumulate

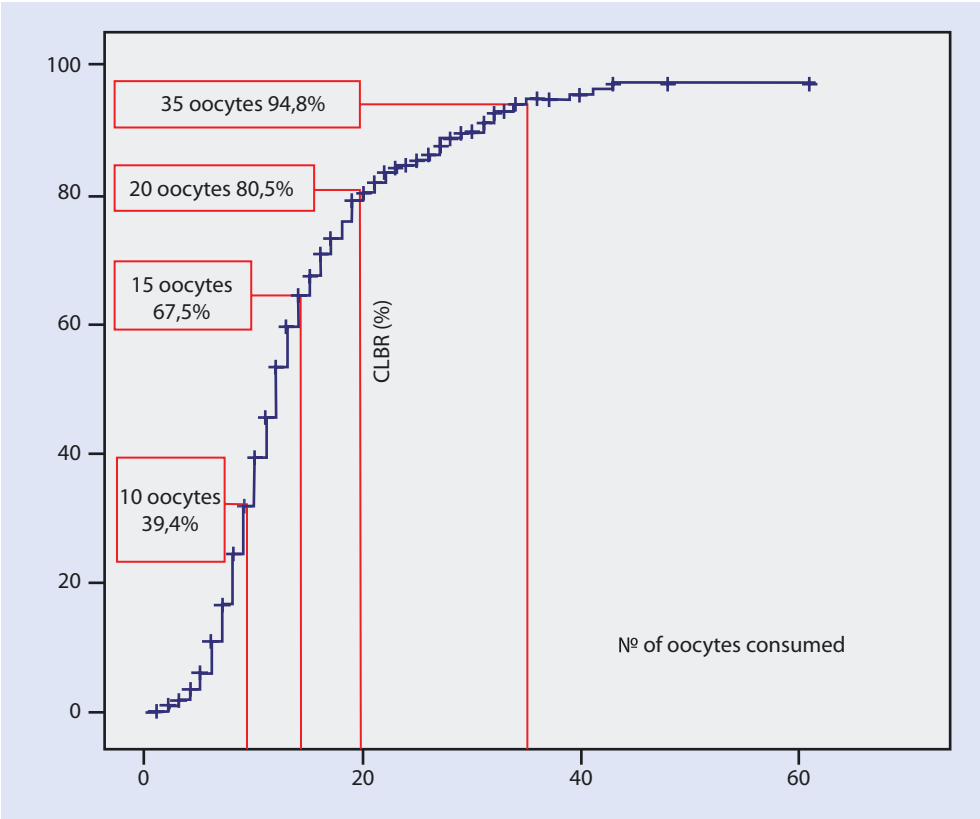


Fig. 55.1 The Kaplan-Meier curve for the cumulative live birth rates (CLBR) of at least one baby, depending on the total number of oocytes consumed

and vitrify mature oocytes; a later IVF cycle successfully ended in a twin pregnancy. Since then, various studies have reported clinical outcomes with cryopreserved oocytes for FP in cancer patients (Table 55.2).

Although FP may initially concern cancer patients, there are many other medical conditions that may compromise fertility, such as endometriosis or high risk for early ovarian failure, where an intervention as safeguarding gametes for future use is required to uphold fertility potential. In addition, age-related decline of fertility is a very common condition in assisted reproductive technology (ART). Elective oocyte vitrification for nonmedical conditions is increasingly accepted as an option to postpone motherhood (Cobo et al. [36]).

We have recently reported, in the largest series to date, our experience in FP in a population of women who electively decided to vitrify their gametes for future use (Cobo et al. [36]).

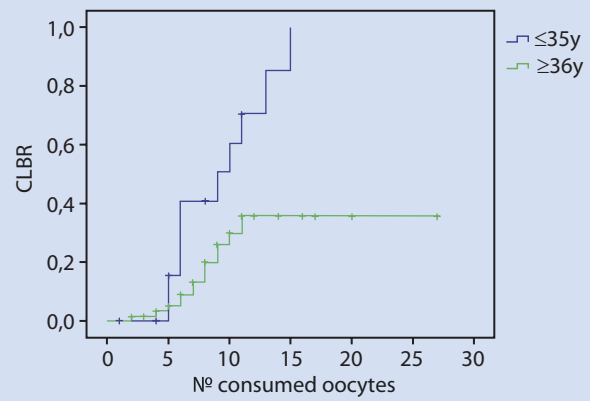
A clear, and expected, effect of female age was observed in our data. Higher outcomes were achieved in women aged ≤35 years. In this group a larger number of oocytes were retrieved and finally vitrified, and the survival and clinical outcomes were equivalent to those achieved in our egg-banking program for ovum donation, with the highest success rates in the youngest group of women (≤29 years). Otherwise, predictably fewer oocytes and worse outcomes were achieved as the age increased, resembling the results of the infertile population of similar age. There is a clear different probability of having a baby according to the number of oocytes consumed when the ≤35-year-old and >36-year-old groups are compared ($P < 0.05$) (Fig. 55.2). Obviously, the more oocytes, the higher the probability, but the relationship is not linear, as shown by the curves, and is strongly related to a powerful confounder, i.e., the age of the patient. When we looked at our data, we observed a huge difference in CLBR when using only five oocytes (15.4%) compared with using eight (40.8%), which means an 8.4% increase in CLBR per additional oocyte if women were ≤35 years old. If they were >36 years old using the same number of oocytes, the increase in CLBR was considerably more modest (from CLBR of 5.1% with the use of five oocytes to 19.9% when eight oocytes were consumed, meaning an increase in CLBR of 4.9% per additional oocyte). With 15 oocytes, the CLBR continued to increase in the ≤35-year-old group, whereas with the same number of oocytes the plateau was already reached in the group of women aged >36 years, meaning that at this point the success is independent from the number of oocytes used up. In light of this, we suggest that at least eight to ten MII oocytes should be vitrified to obtain a reasonable success rate.

55.3.3 Low-Responder Patients

A potential alternative to the management of low-responder (LR) is to create a large stock of oocytes by accumulating vitrified MII oocytes over several stimulation cycles and inseminating them simultaneously. Theoretically, this could

Table 55.2 Live birth reported after fertility preservation in cancer patients: slow freezing and vitrification of oocytes

	Yang et al. [40]	Porcu et al. [41]	Sánchez Serrano et al. [42]	Kim et al. [43]	García-Velasco et al. [44]	Alvarez et al. [32]	Da Motta et al. [45]
Type of malignancy	Hodgkin's lymphoma	Borderline ovarian tumor	Breast cancer	Chronic myeloid leukemia	Non-Hodgkin Lymphoma	Invasive ovarian carcinoma	Breast cancer
Cryopreservation technique	Slow Freezing	Slow Freezing	Combined OTC-SF + OV (Cryotop)	Vitrification (EMG)	Vitrification (Cryotop)	Vitrification (Cryotop)	Vitrification (Cryotip)
Age at FP	27	26	36	22	31	28	36
No. of cryopreserved oocytes	13	7	16	7	4	14	28
Storage time (years)	6	4	2	9	2	1	6
Pregnancy	Single	Twin	Twin	Single	Single	Heterotopic	Triplet
Number of live births	1	2	2	1	1	1	1
Weeks of gestation	37	38	34	35 + 3 days	39	33	–
Weight of babies	3062	2100 and 2400	1650 and 1830	2410	3440	2650	2970
Sex of baby	Male	Females	Males	Male	Male	Male	–



≤35 years old		≥36 years old	
Nº oocytes	CLBR (IC95%)	Nº oocytes	CLBR (95% CI)
5	15,4 (–4.2–35.0)	5	5,1 (–0.6–10.7)
8	40.8 (13.2–68.4)	8	19,9 (8.7–31.1)
9	50,6 (31.6–79.6)	9	25.8 (12.7–38.9)
10	60,5 (34.5–89.5)	10	29,7 (15.2–34.2)
15	85,2 (60.5–100)	11	35,6 (18.4–52.8)

Fig. 55.2 Kaplan-Meier plotting of the CLBR of at least one baby, depending on the total number of consumed oocytes and categorized by age

help to increase the chances of success by "converting" poor responders into a "normoresponder-like" status.

We reported, in 2012, a study aimed to assess the efficiency of this new strategy for managing LR that takes advantage of vitrification as a way of creating larger cohorts of oocytes (Cobo et al. [37]). This study included 242 LR patients (594 cycles) whose mature oocytes were accumulated by vitrification and inseminated simultaneously (LR-Accu-Vit) and 482 patients (588 cycles) undergoing IVF/embryo transfer with fresh oocytes in each stimulation cycle (LR-fresh). The embryo-transfer cancellation per patient was significantly lower in the LR-Accu-Vit group (9.1%) than the LR-fresh group (34.0%). This result showed that this strategy is useful to avoid patients abandoning treatment due to negative results that impair their ability to cope with the situation. Live birth rate (LBR)/patient was higher in the LR-Accu-Vit group (30.2%) than the LR-fresh group (22.4%), which confirmed the efficiency of this method for managing LR patients. The positive effects of this strategy are even more evident when cumulative outcome is considered, which endorses the treatment as a successful alternative for LR patients.

Other authors also reported the evaluation of this form of vitrification in the context of standard infertile patients. Rienzi et al. [38] conducted a prospective randomized sibling-oocyte

study that included 120 fresh and 124 vitrified sibling oocytes from 40 infertile patients (mean age 35.5 ± 4.8 years). The survival rate was 97%, with a fertilization rate after ICSI of 77% (95/124) per warmed oocyte and 79% (95/120) per warmed/inseminated oocyte. Moreover, the proportions of excellent-quality embryos were the same in each group.

In another study, the cumulative outcome after the transfer of embryos derived from fresh and vitrified oocytes from a single ovarian stimulation cycle was calculated. The study included 182 ICSI cycles in which oocyte vitrification was also performed. The cumulative CPR was calculated when after failing a fresh embryo transfer, the patient underwent a second embryo transfer using embryos derived from the vitrified oocytes. Implantation rates in fresh and vitrification cycles were not significantly different for women ≤34 years (Ubaldi et al. [39]).

All these evidences suggest that vitrified oocytes are functionally similar to fresh oocytes in terms of fertilization, development, and implantation potential.

55.4 Practical Aspects of Oocyte Vitrification

55.4.1 Laboratory Procedure

The procedure described below is that developed for Cryotop. However, different methods exist on the market, and it is advisable to follow the manufacturer's recommendations.

- **Required Equipment**
  - Cryotop (Kitazato Biopharma, Tokyo, Japan)
  - Repro Plate (Kitazato Biopharma, Tokyo, Japan)
  - Cooling rack (styrene box for liquid nitrogen)
  - Liquid nitrogen
  - Storage tank
  - Stereomicroscope, stopwatch or timer, tweezers, Pasteur pipette, micropipettes, and tips

- **Solutions:**
  1. Basic solution (BS) is made of Hepes-buffered TCM-199 supplemented with hydroxypropyl cellulose (HPC), only for oocyte vitrification.
  2. Equilibration solution (ES) consists of 7.5% ethylene glycol (EG) and 7.5% dimethylsulphoxide (DMSO) dissolved in BS.
  3. Vitrification solution (VS) consists of 15% EG, 15% DMSO, and 0.5 M trehalose dissolved in BS.
  4. Thawing solution (TS) consists of 1.0 M trehalose dissolved in WS.
  5. Dilution solution (DS) consists of 0.5 M trehalose dissolved in WS.
  6. Washing solution (WS) is made of Hepes-buffered TCM-199 supplemented with HPC.

55.4.1.1 Vitrification Procedure

- **Preparation on the Day**
  1. Bring BS, ES, and VS to room temperature (24–26 °C) 1 h before application.
  2. Fill the cooling rack completely with liquid nitrogen.
  3. Write necessary information about the patient on the handle of the vitrification device.

- **Equilibration Step**
  1. Aspirate the oocytes from the culture dish and keep them at the tip of the capillar or Pasteur pipette, and transfer them within minimum drop volume to 20 µl of BS into a reproplate well (Kitazato, biomedical supply, Tokyo, Japan).
  2. Add 20 µl of ES surrounding the previous drop containing the oocytes (Kitazato, biomedical supply, Tokyo, Japan). Wait for 3 min at room temperature.
  3. Add another drop of 20 µl of ES in the same way as in the previous step and wait for 3 min more.
  4. Add 240 µl of ES slowly circling the previous drops and wait between 6 and 9 min. At the end of this step, the oocytes should be re-expanded and should recover their original appearance.

- **Vitrification Step**
  1. Dispense 300 µl of VS solution into two wells of the reproplate.
  2. After equilibrium, aspirate the oocytes at the tip of the pipette. Afterward, place them on the surface of the VS. Try to carry a minimum volume of ES solution. Note that the oocytes will float due to the high density of the VS media.
  3. Remove the ES medium just placed together with the oocytes (it will be noted clearly due to the difference in density) and wash the pipette outside the VS well. Keep removing continuously and keep on discarding the medium outside the plate. Repeat this operation as many times as possible within 30 s.
  4. Aspirate the oocytes and bring them to the bottom of the plate (the floating will stop as soon as they start to equilibrate with the medium).
  5. Repeat the same procedure in the next VS well. Place the oocytes in the bottom of the plate and move them many times around the well, repeating the washing process during 30 more seconds.
  6. Aspirate the oocytes and maintain them at the tip of the pipette within minimum volume of VS.
  7. Place the Cryotop under the microscope.
  8. Proceed to load the oocytes onto the Cryotop within minimum volume.
  9. Aspirate excess medium to make sure they are contained within minimum volume.
  10. Immerse the Cryotop directly into the liquid nitrogen container.
  11. Place the plastic protector Cryotop.
  12. Transfer the oocytes to the storage tank.

55.4.1.2 Warming Procedure

- **Preparation on the day**
  1. Warm TS vial and a petri dish up to 37 °C at least 1 h.
  2. Bring DS and WS to room temperature (24–26 °C) 1 h before application.

3. Fill the cooling rack completely with liquid nitrogen and retrieve the Cryotop from the storage tank. Keep it submerged in liquid nitrogen.
4. Drop 300 µl DS into first well and 300 µl WS each into second and third well of the repro plate.

Dilution and Warming Step

1. Remove the protective straw maintaining the Cryotops immersed in liquid nitrogen.
2. Remove the Cryotop from the liquid nitrogen container, and submerge it instantly (straight and quick movement) into 4 ml of a solution containing 1.0 M trealose (TS) (Kitazato, medical supply, Tokyo, Japan).
3. Visualize the oocytes and immediately start the countdown to 60 s.
4. Do not manipulate the oocytes within the first 40 s.
5. Retrieve the oocytes from the Cryotop with very gentle manipulations within a minute in TS solution.
6. Transfer the oocytes to the DS well in the reproplate. Aspirate the oocytes very gently, and continue to aspirate TS until the column reaches approximately 2 mm in length.
7. Take the pipette to the DS well and immerse it to the bottom. Dispense the 2 mm column at first. The column of TS within DS will be clear due to the difference in density of both. Leave the oocytes on the "top" of this column. Wait for 3 min.
8. Transfer the oocytes to well WS exactly as in the previous step. Wait for 5 min.
9. Transfer the oocytes to the next well of WS well. Leave the oocytes on the surface of this media; they will drop down immediately due to the absence of differential density between media. Wait for 1 min.
10. Transfer the oocytes to the regular culture dish and place them in the incubator at 37 °C. wait for 2 h before ICSI.

55.5 Equipment and Facilities

All our samples are cryopreserved by vitrification. This procedure, as currently performed, is entirely manual-operated, making not necessary the use of any equipment to carry out the vitrification process itself. The ease and efficiency of vitrification have been a turning point in the field of cryopreservation, making the whole process no longer than 20 min (vitrification and warming) involving very simple tools. However, the fact that the samples are vitrified mostly contained in very low volumes represents a challenge for further handling, storage, and maintenance of the vitrified samples.

The storage vessel can be the traditional liquid nitrogen tanks or vapor tanks. In our oocyte-bank facilities, we use vapor phase storage tanks (CBS V1500; Custom Biogenic Systems, Michigan, USA), which consist of an outer jacket with liquid N2. This area is responsible for cooling the storage area where the oocytes are maintained in a N_2 gas atmosphere. The cold spreads from the vacuum-insulated jacket by convection and through vents in the storage compartment that expel the nitrogen vapor downward to the bottom of the freezer, thus creating a flow of extremely cold air through the entire storage area. The exceptional uniformity of temperature allows the whole storage tank to be used, achieving temperatures below −180 °C at the upper level and −195 °C at the bottom. Samples can be manipulated in safe temperature ranges (−180 °C) thanks to the working area located on top of the storage area, thus avoiding any risk of accidental warming. An additional advantage of this storage system is that the supply of liquid nitrogen can be programmable, although it also can be performed manually. We have demonstrated the effectiveness of this storage vessel as a strategy for preventing the risk of cross-contamination due to direct contact with the liquid nitrogen (LN), showing comparable results between oocytes vapor-stored versus those stored in conventional LN tanks [31].

A temperature monitoring system is strongly advised as a part of the routine quality control (QC) of the cryolab. In our facilities, we use a system that allows continuous monitoring of the temperature of every storage tank in our unit (DataCare, ControlTemp, Barcelona). The system is able to provide numeric and graphic records and to display alarms in real time with updates every second. A record of incidents occurring during the alarm can be also easily assessed differentiating between active alarms or alarms that were active but are no longer in that state. In case of alarm, the system sends alerts and warnings messages to the authorized personnel.

On the other hand, in order to ensure efficiency, only highly skilled professionals who have to overcome a long learning curve should perform vitrification. Therefore, an adequate learning curve is also one of the most important requirements when performing vitrification that requires close attention. Our training program has produced satisfactory results since the introduction of vitrification in our clinical setting. It consists of different phases that gradually increase in difficulty. To pass to the next level, trainees must acquire the necessary skills as well as achieve a preset survival rate.

Review Questions

1. What are the reasons responsible for the ability of vitrification to rule out chilling injury and the crystallization of the cytoplasm?
2. What role does the cooling and warming rate play in vitrification protocols?
3. Which population can benefit from oocyte vitrification?
4. How can the toxicity of cryoprotectants be reduced in vitrification protocols?
5. What are the main benefits of egg-banking for ovum donation programs?
6. What factors are mainly responsible for success in the elective fertility preservation?

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Slow Freezing of Embryos

Liesl Nel-Themaat, Ching-Chien Chang, Thomas Elliott, Diana P. Bernal, Graham Wright, and Zsolt Peter Nagy

This chapter is dedicated to the late Dr. Stanley Leibo. May you rest in peace knowing how many lives you touched both in the fresh and frozen state.

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Learning Objectives

- Historical overview of embryo cryopreservation
- Mechanisms of freeze-thaw damage during embryo freezing
- Components of slow embryo freezing procedures
- Factors that determine embryo freeze/thaw success rates
- Applications and impact of embryo freezing in assisted reproduction
- Open vs slow cryostorage systems
- Differences between slow freezing and vitrification
- Legal and ethical considerations for freezing of embryos
- Future of slow freezing in embryo cryopreservation and assisted reproduction

56.1 Historical Overview of Embryo Cryopreservation

Cryobiology (the study of life at low temperature), as we know it today, has come a very long way to where we now can freeze and thaw living cells while maintaining viability, without any apparent detrimental effects. Currently, cryopreservation plays such a significant role in most biological fields that it is hard to imagine scientific progress without it. Examples range from the use of cryopreserved bacteria for genetic engineering, preservation of frozen tissues for transplantation, the large number of cell culture studies for disease research to assisted reproduction in humans and animals. To embark on this voyage back in time, one must consider the founding principles that led to the successes that were ultimately reported during the first half of the twentieth century. It follows a condensed representation of the history of modern cryopreservation but a more detailed review can be found in [1].

The start of this exciting journey goes back more than 300 years, to the early experiments by Robert Boyle, who was studying the effects of temperature on the volume of gasses. The foundation laid through his work led to the first Nobel Prize in chemistry, awarded to Jacobus van't Hoff, for his groundbreaking work on laws of chemical dynamics and osmotic pressures in solutions. The Boyle–van't Hoff plot still plays a fundamental role in cryobiology, as a means to describe the effect of osmotic pressure on cell volume. (The significance of these osmotic principles during freezing will be discussed in subsequent sections.)

During the same era, liquefaction of oxygen, hydrogen, and nitrogen was developed by French physicist, Louis-Paul Cailletet, which later allowed ultra-cooling of specimens. The importance of this discovery for modern-day cryobiology goes without saying.

As interest in life at low temperatures grew, so did the number of publications on the effect of cold on various biological systems. Most noteworthy of these is the work of a Swiss-born Priest named Basile J. Luyet, whose studies are best described by the title of his classic monograph "Life and Death at Low Temperatures." He proceeded to be the founder of the Journal *Biodynamica* and first president of the Society for Cryobiology. Father Luyet is thus generally known as the father of cryobiology.

The next decades saw major developments in low temperature biology, especially with spermatozoa preservation. In fact, the first observations on cooled spermatozoa occurred in 1866 by Italian military physician, P. Mantegazza, who noted that human spermatozoa became immotile when cooled in snow. He further envisioned the possibility for a deceased soldier to father a child by cooling sperm to maintain viability.

Mutual investigations by John Hammond and Arthur Walton on ejaculated and non-ejaculated sperm, respectively, showed that temperature affects ejaculated and non-ejaculated sperm differently, and that cooling of the latter could preserve its fertilizing capacity. We now know that capacitation is induced by exposure to seminal plasma, which changes membrane properties and renders spermatozoa more susceptible to temperature damage. Interestingly, Hammond predicted that "in these days of rapid aeroplane transport, it might be possible to move entire herds of animals around the world from chilled samples of semen" less than 30 years after the Wright brothers' first flight. So progressive was his thinking, that his vision today is a reality with human and animal gametes and embryos that are transported large distances, often across continents and recently even into space!

With the artificial insemination field taking off around 1930, the possible impact that the ability to freeze semen long term became widely recognized, motivating several groups to attempt long-term storage of semen by freezing in liquid gas. Success rates varied. It is notable that in 1938, Luyet and Hodapp described an experiment where spermatozoa survived vitrification in liquid air when exposed to a 2M sucrose solution. Although about 40% initially lost their motility during incubation in the hypertonic solution, cells that survived the incubation and were vitrified and warmed survived.

Despite advances, post-thaw survival rates remained typically low, until the accidental breakthrough discovery of the cryoprotective properties of glycerol by Christopher Polge and Audrey Smith under guidance of Alan Parkes in 1947 while working on fowl spermatozoa [2]. (It should be noted that in the previous year, Jean Rostand found that addition of glycerol protected frog spermatozoa from freeze damage. Fertilizing capacity of thawed samples, however, was never confirmed. Furthermore, there are unverified reports of using glycerol as a cryoprotectant for the freezing of mammal and bird semen in Russia). Polge's discovery was followed by successful freeze-thaw of spermatozoa in various mammalian species [3, 4]. Six years later, Bunge and Sherman [5] reported three human pregnancies resulting from artificial insemination with frozen sperm [5].

The second half of the twentieth century saw great improvements and refinement of the technique, while a wealth of information on freezing principles was produced. This included the necessity of water removal and the addition

of cryoprotectants and macromolecules to prevent intercellular ice crystal formation and membrane damage. We will elaborate on these topics in subsequent sections.

The next challenge investigators were facing was that of freezing the female gamete and embryo. At the end of the 1940s, M.C. Chang, who was working under Hammond at the time, started experimenting with cooling embryos and found that while slow-cooled embryos survived, rapidly cooled ones did not (Chang 1948 [6–8]). He proceeded to produce normal rabbit offspring from zygotes, cleavage and blastocyst stage embryos, all stored for at least 24 h at either 10 °C or 0 °C [8, 9]. Chang and Smith both followed these findings by experimenting with rabbit oocytes [10, 11]. Shortly after, Sherman and Lin reported birth of mouse pups from frozen-thawed oocytes that were transferred back into recipient females before being mated. These and other early studies revealed that oocytes are much more sensitive to low temperatures than embryos. During years of subsequent research, the reasons for this difference in sensitivity between oocytes and embryos were investigated but are beyond the scope of this chapter. For more information on the topic, see [12].

During the late 1950s and early 1960s, Peter Mazur turned his attention toward elucidating mechanisms involved in cell damage during freezing and thawing. His discoveries (discussed in subsequent sections) played a seminal role in the work by Whittingham et al. [13] that led to the first truly successful and repeatable freezing and warming of mouse embryos, as indicated by their high birth rate after transfer [13]. This was followed by more successes in other mammalian species [14, 15] and finally human embryos in the early 1980s [16]. Thirty-five years later, embryo cryopreservation has become an integral part of clinical embryology. The large collection of data available demonstrates that there is no single optimum technique for slow freezing and thawing. Instead, numerous different combinations of cryoprotectants, cooling and warming rates can lead to success. One thing that all these techniques do share is that they stem from the principles laid out through the diligent work of the cryobiology pioneers.

Since the first edition of this textbook almost 6 years ago, a drastic shift has occurred in the field, in that the vast majority of clinical reproductive labs now exclusively use vitrification as their cryopreservation platform. The reason for this preference of vitrification over slow freezing is not only based on perceived improved outcomes (although many studies failed to show statistical superiority of vitrification over slow cooling [17, 18]) at both cleavage and blastocyst stage cryopreservation observed following vitrification [19, 20] but also due to the significantly less time-consuming nature of vitrification protocols compared to that of traditional slow-freezing protocols. In fact, many commercial manufacturers have stopped producing their slow-freezing solutions altogether and are only selling thaw solutions, as many programs still have to thaw embryos that were frozen by slow cooling in the past on a regular basis. Therefore, knowledge of the technique is essential for any clinical embryologist.

56.2 Mechanisms of Freeze/Thaw Damage

Cells are composed of 60–85% water and a mixture of solutes and suspended particles, such as proteins, nucleic acids, and lipids. Various studies have shown that about 10% of the water in cells are bound to cellular components, rendering it incapable of freezing (Reviewed by [21]). As freezing occurs, the "free" water is removed from cells either by crystallization inside the cell or by moving out of the cell via osmosis through the semi-permeable membrane (due to hypo-osmotic conditions that occur with extracellular ice crystal formation) to crystallize extracellularly. Intracellular ice formation is the most likely cause of cryodamage, since it can rupture the cell membrane and displace or disrupt organelles. Therefore, it is desirable to dehydrate cells of their "free" water prior to freezing. This outward osmosis can be facilitated by using hypertonic freezing solutions and slow-cooling rates, which gives the water time to exit the cell as extracellular ice formation progresses. A direct consequence of dehydration is cell shrinkage, as the cell loses a significant part of its volume. In fact, cells will maintain equal chemical potential of the water inside and outside of the cell and therefore the cell volume is (in most cases) inversely proportional to the external osmolality [21]. Too rapid dehydration can cause physical stress on the membrane as it conforms to the reduced cytoplasmic volume. The same is true for too rapid rehydration, where cells swell up too rapidly and can literally burst like a balloon. This happens because during osmotic shrinkage, hydrostatic pressure draws some extracellular solution containing permeating molecules into the cell to replace the water volume that is removed. Thereby, some of the physical stress on the membrane is relieved. Thus, if a cell is inserted into a hypertonic solution, one will witness shrinkage followed by re-expansion to its original volume, as long as the solutes responsible for the high extracellular osmolarity are membrane permeable. When rehydration occurs too quickly, these replacement solutes may not have enough time to leave the cell as water moves back in before the critical volume is reached and the cell membrane gets overstretched, causing the cell to burst.

Membrane damage during freezing also occurs when intracellular ice crystals form before complete shrinkage of the cell. As ice forms, solutes in the remainder of the intracellular water cause its freezing temperature to decrease, thereby allowing further dehydration and cell shrinkage and the membrane closing in on the sharp ice crystals. This can be detrimental, since ice crystals can puncture the membrane as the cell volume continues to decrease. It is therefore crucial to not only remove as much water as possible prior to freezing but also to allow complete equilibration of the extracellular and intracellular solutions to increase the volume that will remain inside the cell and prevent the membrane from closing in on the ice crystals. This can be accomplished by high concentrations of permeable solutes in the freezing medium combined with a slow-freezing rate.

Another proposed source of freeze damage is excessive dehydration that occurs as the osmolarity of extracellular fluid increases when water is removed by ice formation. Some studies suggest that there is a maximum level of shrinkage that a cell can withstand before being damaged, and when this point is superseded during freezing, cell injury occurs [22].

There are indications that cryodamage can also result from the increased concentration of electrolytes that accompanies the "free" water crystallization, independent of the physical damage by ice. Basically, the same mechanisms that cause red blood cells to hemolyze at high salt concentrations apply. A review about this mode of damage and its variants can be found in [23].

Apart from the above discussed bursting of cells that dehydrate too quickly, thawing presents another set of challenges. In fact, some studies suggest that the majority of cryoinjury occurs during the thawing rather than the cooling process, due to recrystallization of small ice crystals into larger ones [21]. This is especially true with slow rates, which allow enough time for this ice crystal growth.

Much disagreement exists on which is the best method of thawing, which essentially represents the rate of warming. However, published works demonstrate that the ideal warming rate greatly depends on, and is proportionate to, the cooling rate that was used during freezing. Furthermore, the cell type, cryoprotectants used, and freezing technique also affect the warming method that will lead to the highest post-thaw survival rates.

56.3 Components of Slow Embryo Freezing

56.3.1 Cryoprotectants

Cryoprotectants are molecules that prevent cryodamage by reducing intracellular ice crystal formation. Their sole purpose in freezing protocols is to protect the cells from cryodamage by preventing the events described above. Two types, permeating and non-permeating cryoprotectants, are typically used in combination to dehydrate (non-permeable) and equilibrate (permeable) cells prior to freezing. The rate of diffusion of permeable cryoprotectants into the cell is slower than the rate of water exiting through osmosis, which necessitates some time for equilibration until the original cell volume has been restored. Once inside the cell, these agents prevent ice formation by lowering the freezing temperature of the cytoplasm. There are additional theories regarding the mode of protection of cryoprotectants, with strong arguments for all of them.

Some studies suggest that permeable cryoprotectants act as replacement diluents to keep relative electrolyte concentrations low when water is removed from the cytoplasm or freezing medium, thereby protecting them from high solute concentrations [24]. Others argue that they substitute the lost water fraction to keep shrinkage to a minimum and increase the unfrozen volume of the cell during freezing and thawing. Yet another school of thought is that cryoprotectants interact with phospholipids in the membrane bilayers to make them more stable. Then there is the fact that cells frozen in media without cryoprotectants are pushed into channels between forming ice crystals, while those that contain cryoprotectants in the media are encapsulated within the ice, with the latter scenario apparently less damaging. Despite the disagreements about the exact modes of protection, it is agreed that these agents are an essential component of freezing solutions. The usability of each ingredient depends on its toxicity to cells, which played a big part in optimization of freezing media for specific cell types.

Today, the most commonly used ingredients for slow-freezing human embryos are permeating 2,1-propanediol and dimethylsulfoxide for cleavage stages and glycerol for blastocysts, whereas sucrose is typically the non-permeating additive. An extensive overview of the various available cryoprotective agents and their use can be found in [25].

56.3.2 Media Formulations and Procedures

As discussed above, numerous different cryoprotectants can assist in protecting embryos against cryodamage when used appropriately. Consequently, there are various medium formulations that can lead to success. Typically, freezing medium consists of a base medium, such as M199, buffered with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and supplemented with either one or a combination of permeating and/or non-permeating cryoprotectants. Commercial freezing media companies keep their formulations confidential, and they may be based on more complex media corresponding to the rest of the manufacturer's media series. However, the basic formulations of commercial brands are pretty standard and all based on published slow-freezing/thawing formulations as described above.

Conditions exerted on the embryo by these media can be detrimental if they are not introduced slowly. Therefore, cryoprotectant concentrations are usually increased systematically via a stepwise equilibration technique, allowing water to exit the cell and simultaneously allow cryoprotectants to diffuse into the cell gradually. Usually, the first solution will contain permeating cryoprotectants such as propylene glycol and DMSO, while the final freezing medium will contain a higher cryoprotectant concentration (1.0–1.5 M) and sucrose (0.1–0.3 M). Some protocols, however, use a one-step equilibration method with equally successful results, but its effectiveness depends on the species and embryo stage. Equilibrium is reached when the osmotic and chemical gradients caused by water and cryoprotectants inside and outside the cell membrane disappear. Equilibration and loading are then usually performed at room temperature to minimize cytotoxic effects of the cryoprotectants.

Once equilibration is complete, usually after 5–15 min, the embryos are loaded into a cryopreservation carrier. For slow freezing, the most commonly used devices are plastic straws that can be heat sealed after loading. They typically accommodate 0.25–0.5 mL of medium in which the embryos are suspended. It is a standard procedure to include two small air bubbles, one before and one after the section that

will include the embryos, to assist in locating the embryos upon thawing. Cryogenic vials, similar to those used for freezing somatic cells, as well as glass ampoules, have also been used successfully. Each of these has been designed to fit into an automatic, controlled-rate cooling device that carefully regulates the rate of cooling. This, however, can also be achieved by carefully layering the straws/vials at a specific distance above liquid nitrogen vapor. Naturally, the latter is less precise and much more labor intensive.

Initially, the freezing solution containing the embryos is cooled from room temperature, at a rate of 1–3 °C/min, to a slightly super-cooled state between −5 and − 7 °C. Then, seeding is initiated either manually or automatically to start crystallization in a controlled manner and prevent further super-cooling. Subsequently, the cooling process is maintained at a reduced rate of 0.3–0.5 °C/min to below −30 °C. Finally, the embryos are cooled to −180 °C at 40 °C/min before submersion in liquid nitrogen. Some protocols omit the final cooling phase and require liquid nitrogen submersion once the embryos have reached temperatures below −30 °C.

The thawing procedure can be as detrimental to embryo survival as freezing, since re-crystallization may occur in the small time window during which the liquid will transition through the melting temperature. To prevent this, high warming rates are applied. Typically, straws, vials, and ampoules are thawed either in air at room temperature or in a warm water bath at 37 °C. Once the freezing solution is completely melted, embryos are removed from the carrier and transferred into a diluent solution. This usually consists of the same medium used for freezing but without the permeating cryoprotectives and the same concentrations of sucrose to remove the permeating cryoprotectants. Rehydration is then achieved by decreasing the sucrose concentration systematically, usually in one or two steps, until all sucrose is removed from the medium. Embryos are subsequently returned to the incubator for conventional embryo culture after the final wash.

Immediate evaluation of survival is achieved visually by assessing the status of the cell membranes. In cases where embryos are cultured after thawing, the developmental competence and blastocyst re-expansion could be indicative of a successful freeze-thaw.

56.4 Factors that Determine Success

56.4.1 Embryo Stage

Over the years, research has indicated that embryos at different stages have different tolerance to cryopreservation. The reasons why embryos sometimes survive freeze-thaw better at one stage than another is not clear. Instinctively, from what we know of the effect of surface-volume ratio on cryopreservation success (for review, see [21]), we may assume that blastocysts will be most tolerant, followed by morulae, cleavage stage, and lastly zygotes, based on the size of the individual blastomeres at these respective stages. However, data clearly showed that embryos at the two-pronucleus (2PN) stage are no less tolerant to freezing and thawing than any other stage [26–28]. This becomes more baffling when one considers how challenging oocyte freezing has proven to be. Clearly, the difference in freeze tolerance encompasses more than just the volume of the individual blastomeres. So far, there is no single embryonic stage that is definitely superior in terms of outcome for a frozen embryo transfer (ET) cycle [29]. The parameters associated with assessment of success in embryo freezing are predominantly survival, subsequent development, and implantation. However, results were inconsistent when the survival rates of pronuclear versus multicellular (Day 2 and Day 3) stage frozen-thawed embryos were compared [27, 30]. Therefore, there is still no consensus as to which embryonic stage is superior for freezing tolerance, and the decision of freezing stage now primarily depends on the IVF program's management and individual patient cycle parameters. Nonetheless, it is beneficial to know the advantages and disadvantages of all embryonic freezing stages, which will be discussed below.

56.4.1.1 Pronuclear Stage

Slow freezing at the pronuclear stage has been widely used to cryopreserve human embryos, because it gives consistent survival and subsequent development after thawing [26–28]. One major advantage is that the possibility of future embryo biopsy and/or transfer at any stage is reserved when embryo freezing is performed at the pronuclear stage. The pronuclear stage embryo, however, has only one cell, which means embryo survival is either all or nothing. Furthermore, embryo quality cannot be assessed before freezing like in the case with more advanced stage embryo freezing, since the only measurable quality parameter at this stage is the presence or absence of pronuclei, and zygote scoring may not be as predictive as cleavage or blasotcyst-stage assessment. After thawing, pronuclear stage embryos are typically cultured for at least one, but usually more days to Day 3, 5, or 6, to obtain more data for embryo selection prior to ET. This requires a good number of embryos, enough to obtain the desired number of acceptable blastocysts for ET and potentially refreezing.

56.4.1.2 Cleavage Stage

Traditionally, the majority of embryo cryopreservation was performed using propanediol and sucrose to slow-freeze at the cleavage stage [31]. Survival rates of 60–88% were reported [32–34]. At cleavage stages, the widely accepted criterion for embryo survival in a clinical situation is survival of a minimum of 50% of the original blastomeres. Often, some necrotic blastomeres are present after thawing due to cryodamage. It was therefore suggested to remove those necrotic cells before subsequent culture or ET. We will discuss lysed cell removal (LCR) in subsequent paragraphs. In summary, the main advantages of freezing at the cleavage stage are that embryo cleavage potential has been confirmed before freezing, while cryodamage can be confined to individual blastomeres instead of the entire embryo, as would be at the 2PN stage. Furthermore, it preserves the opportunity to do a

Day-3 biopsy for genetic screening. It should be mentioned that the past few years have seen a dramatic shift in clinical IVF practices to predominately performing single blastocyst transfer, often in conjunction with blastocyst biopsy for preimplantation genetic screening and vitrification, and subsequent frozen-thawed embryo transfer of only euploid embryos.

56.4.1.3 Morula Stage

From all the preimplantation developmental stages, the morula has received the least amount of attention for cryopreservation and ET. However, reports of good outcomes with these procedures at the morula stage exist and the advantages cited for performing them at this stage include better selection than cleavage stages and safer assisted hatching due to the large perivitellin space [35–37]. The reason for the neglect is not due to poor results but due to its intermediate nature. Embryos are typically transferred on Day 3, at the cleavage stage or on Day 5 at the blastocyst stage. Whether embryos will be transferred on Day 3 depends on the number and quality. The advantage of extended culture is the ability to select embryos more extensively based on their developmental and differentiation potential, as well as the ability to do trophectoderm biopsy, which has become the preferred biopsy stage. However, if a patient has too few embryos or they are of suboptimal quality, Day 3 transfers may still be performed. Therefore, should embryos be assigned for Day 5 transfer, there are probably enough good quality embryos to culture all the way to the blastocyst stage, which has better selection criteria than the morula stage. It is also beneficial to reduce handling and disturbance of the culture conditions, so quality assessment on Day 4 is omitted. Thus, although rarely used, morula stage cryopreservation is an option for clinics and its utilization depends on the transfer scheme of the individual clinics.

56.4.1.4 Blastocyst Stage

Although the early successes in embryo cryopreservation were achieved at the blastocyst stage [38, 39], embryo freezing at this advanced stage was not routinely used until recent years when blastocyst culture systems became optimized. Culturing embryos to the blastocyst stage creates more selection criteria for ET and cryopreservation, due to prolonged monitoring and a more differentiated state. It further allows IVF programs to maintain their pregnancy rates despite transferring fewer embryos [29] by elective single embryo transfer often paired with preimplantation genetic testing (PGT). Even though embryos could be selected before freezing, the degree of cryodamage that may occur is hard to predict. Resumption of development is also difficult to assess in cryopreserved blastocysts, since any increase in the number of cells is very difficult to verify, and the timeframe of re-expansion for blastocelic activity assessment varies greatly. Also, due to the epithelial-like nature of the trophectoderm and compacted blastomeres of the inner cellular mass, necrotic cells are more difficult to remove from the blastocyst, which may further impede normal development. Nonetheless, blastocysts have 5–6 days of developmental history that can help to determine, with post-thaw morphology, the overall quality of the thawed embryo.

56.4.2 Embryo Quality

Several studies have shown a correlation between embryo quality and freeze tolerance. Typically, cleavage stage cell divisions are synchronized, resulting in an exponential cell number at any point before the morula stage. Non-exponential blastomere numbers are therefore indicative of asynchronous divisions, fragmentation, or degeneration of some of the blastomeres, which are all indicators of inferior embryo quality. Correspondingly, it was shown that embryos with 3, 5, 6, and 7 cells had significantly lower post-thaw survival rates than 2-, 4-, and 8-cell embryos [40–42]. A similar observation was made in the bovine model, where blastocysts of high quality survived freezing better than those of lower quality [43]. Interestingly, Check et al. [44] reported that four-cell embryos had a similar freezing tolerance to six- to eight-cell embryos, suggesting that a slower developmental rate is not included in the quality parameters that affect freeze survival [43]. Although the above-mentioned studies indicate that lower grade embryos are less prone to survive freezing, embryologists may still choose to freeze these inferior embryos to maximize the patient's number of transferable embryos in a given cycle, especially if the other quality parameters are acceptable for transfer before freezing.

56.4.3 Lysed Cell Removal

At the cleavage stage, the widely accepted clinical criterion for embryo survival is a minimum of 50% survival of the original blastomeres. However, necrotic blastomeres resulting from cryodamage are often present after thawing in embryos that are still in the acceptable survival range. These blastomeres can be removed using a technique called lysed cell removal (LCR), during which the cryodamaged blastomeres are aspirated from the embryo (see ◘ Fig. 56.1). One study found that after cryopreservation "the percentage of gestational sacs with fetal heartbeat obtained after transfer of fully intact embryos was almost three times higher than that after transfer of partially damaged embryos" [45]. The authors speculated that lysed blastomeres might produce factors as they degrade that are detrimental/toxic to undamaged cells. Additionally, lysed cells could potentially disrupt cell-cell communication between viable blastomeres. Studies in the mouse model demonstrated that LCR can restore developmental potential [46] and promote frozen-thawed embryonic development [47]. Lysed cell removal has also been used following human embryo cryopreservation at multicellular stages, and results showed that it could significantly improve clinical outcomes [48–50]. It should be noted that in the mentioned experiments, assisted hatching was performed in conjunction with LCR, a practice that was also

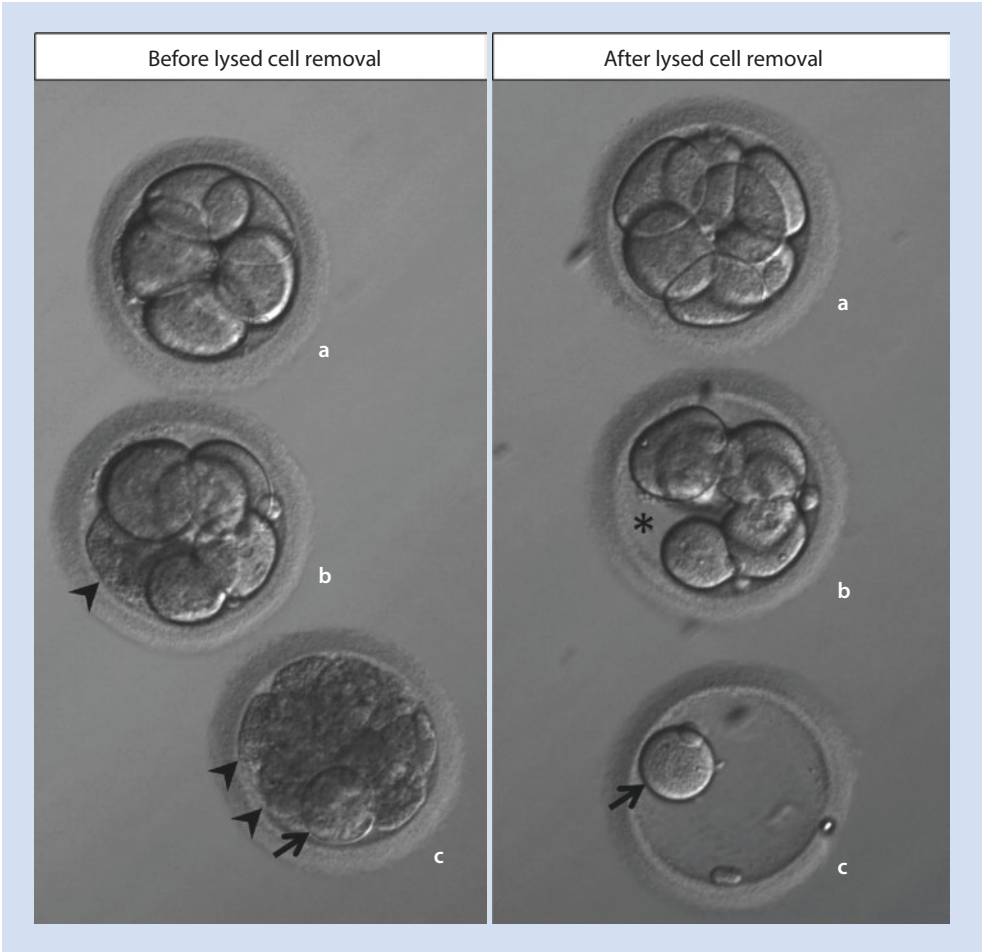

Fig. 56.1 Cleavage stage (Day 3) human embryos after slow freezing and thawing. Three embryos (**a**–**c**) are shown before (left panel) and after (right panel) lysed cell removal (LCR). Embryo **a** had no lysed cells after thawing. Embryo **b** was partially lysed (arrowhead), and these cells were removed as seen in the space present after LCR (asterix). Since only one or two cells were lysed (<50%), embryo **b** survived and is suitable for transfer. Embryo **c** had only one intact cell (arrow) after thawing, while the rest were all lysed (arrowheads) and removed. Embryo **c** therefore did not survive freezing and thawing and is not suitable for transfer

shown beneficial after vitrification and warming [51] and will be discussed in the next section. In conclusion, removal of degenerated blastomeres is a relatively simple procedure that can significantly impact implantation and pregnancy rates after cryopreservation, especially with slow freezing.

56.4.4 Assisted Hatching

Several reports have claimed that assisted hatching by thinning, breaching, or completely removing the zona pellucida prior to fresh ET significantly improves implantation and chemical pregnancy rates (for recent review, see the publication by The Committee of the Society for Assisted Reproductive technology and the Practice Committee of the American Society for Reproductive Medicine [52]). Various effective techniques have been described, including acid Tyrode's solution drilling/thinning [53], mechanical partial zona dissection [54], laser drilling [55], piezo drilling [56], enzymatic zona digestion [57], and most recently mechanical zona expansion through hydrostatic pressure [58]. Although the exact alterations that occur in the zona ultra-structure as zona hardening occurs remain elusive, oocyte exposure to cryoprotectants and freezing was shown to induce cortical granule exocytosis, zona delamination, and zona fractures [59, 60]. Similar structural changes may occur during embryo freezing, and these alterations in zona structure may prevent zona thinning and subsequent embryo hatching. Studies have demonstrated that assisted hatching following cryopreservation indeed improved implantation and pregnancy rates [61, 62]. Therefore, it is a common practice to perform assisted hatching in post-thawed embryos. Although conflicting study results and disagreement exist on the benefit for fresh ETs, the most common opinion currently is that the possible advantages of employing it for frozen-thawed ETs exceed its minute risks, and thus assisted hatching is typically performed on frozen-thawed embryos.

56.4.5 Additional Considerations

Several other factors may affect embryo survival after freezing, and those discussed below are by no means exclusive.

The technique used during execution of the freezing protocol may severely affect the success rate. One example is performing the stepwise equilibration method in a timely manner with gentle handling of the embryos to prevent mechanical damage during loading. It is thus important that technicians are properly trained to perform all the procedures the correct way.

When embryo biopsy is performed for preimplantation genetic diagnosis, embryos are subjected to crude mechanical

disruption, especially in the case of blastocysts. Although the majority of embryos recover from well-executed biopsies, the overall embryo tolerance is affected, thereby decreasing its likelihood to survive freezing and thawing [63]. However, when patients order genetic screening, embryologists have no choice but to perform biopsy and subsequent cryopreservation. Therefore, each case should be considered individually based on the total number and quality of embryos, as well as the importance of the genetic diagnosis.

Transportation of embryos has become an integral part of our field, not only to move embryos with a patient, if they relocate and switch clinics, but also for donation and adoption programs. Proper transportation technique that insures that embryos are not exposed to temperatures that can alter the frozen/vitrified stage is of utmost importance for subsequent survival during thawing. One major benefit of slow freezing is a higher tolerance to accidental warming, due to the significantly larger volume embryos are frozen in, as well as larger devices that can better maintain temperatures during fluctuations. This may be a consideration for embryo and gamete banks that ship large numbers of embryos across the nation and/or globe.

56.5 Applications and Impact

Cryopreservation of human embryos was introduced more than three decades ago, mainly as an approach to save supernumerary embryos produced and stored after ovarian hyper stimulation [16, 38, 39, 64]. The Society for Assisted Reproduction Technology (SART) and the European Society of Human Reproduction and Embryology (ESHRE) both continuously publish statistics on embryo freezing and birth rates in the USA and Europe, respectively. These are valuable data sources for seeing the overall impact of cryopreservation on clinical infertility treatment. However, the data does not distinguish between methods of freezing, rendering it impossible to determine the current success rates resulting from slow freezing alone or vitrification. Moreover, with the rapid decline in utilization of the somewhat outdated slow-freezing procedure, it is unlikely that we will see a significant number of publications on slow cooling in the future. Since vitrification is becoming more widely used, the weight that slow freezing has on overall IVF cryopreservation outcomes is likely decreasing significantly each year, as more clinics are switching over to vitrification for reasons discussed below. Therefore, we will briefly look at the impact of embryo cryopreservation as a whole.

In Europe, ESHRE reported almost 460,000 in vitro cycles in 2006, of which more than 15% were frozen ETs [65]. In 2012 (the latest available data), these numbers increased to 22% frozen transfers of over 640,000 cycles [66]. In the USA, since the publication of the first edition of this textbook, SART data indicated that although the percentage of FETs has remained roughly unchanged at about 18%, the total number of reported IVF cycles has steadily increased annually to about 188,000 cycles in 2014. About 5000 embryo banking cycles for fertility preservation were also performed (► www.sartcorsonline.com). Since many practices currently still use slow freezing and most practices perform slow-cooling procedures on a regular basis from embryo donations or embryos frozen in prior years, the impact of this technology on infertility treatment is still relevant.

One of the most common reasons a patient may opt for embryo freezing is to preserve excess embryos that were not transferred during the fresh cycle. The advantage of this is that the patient's chances of getting pregnant from a single egg retrieval significantly increases as she can have multiple embryo replacements without having to transfer more than one embryo in a single transfer. This reduces her risk of a multiple pregnancy and thus various complications.

A major drive toward elective single ET (eSET) in the field is further increasing the importance of embryo cryopreservation, allowing the patient additional opportunities for pregnancy should her first eSET not result in one. Furthermore, legislation in some European countries limits the number of embryos that can be transferred. In Germany, for example, it is illegal to culture more than three embryos from one cycle at a time, and every generated embryo must be implanted. Freezing extra embryos at the 2PN stage provides an alternative method to utilize the retrieved eggs to its full potential instead of only continuing with three fertilized eggs, as 2PNs are not considered embryos. Embryo freezing thus provides a way to store the extra embryos and makes it unnecessary for the patient to go through an entire stimulation and retrieval again and again.

Another instance in which a patient may want to freeze embryos is when the ET is cancelled due to hyperstimulation. Historically, before freezing became available, this condition required cancellation of the entire treatment cycle and loss of the embryos. Since effective embryo freezing became an option, the patient can simply return in subsequent months for frozen embryo replacements without feeling tempted to put herself at risk by having a transfer anyway.

Preimplantation genetic testing (PGT) has become a routine procedure with many clinics performing it on 100% of their patients (for a recent review, see [67]). The rapidly growing stage of choice for biopsy is Day 5/6 blastocyst, although many labs still perform day-3 blastomere biopsies, likely due to the requirement of a laser system for trophectoderm biopsy, which is a significant expense for smaller IVF labs. Although some genetic reference labs offer results in time for a fresh Day-6 transfer following a Day-5 trophectoderm biopsy, the vast majority of PGT cycles include freezing of all embryos following biopsy.

Due to a significant increase in successful eSETs coupled with highly efficient cryopreservation techniques, more and more patients may have embryos left over after delivering the desired number of babies. Many patients have ethical objections to discarding remaining embryos. A viable option for these patients is frozen embryo donation. In doing so, those patients who failed to conceive naturally or by in vitro

techniques, while eliminating the financial burden of storing embryos. Several embryo donation and adoption agencies exist, which include embryos frozen years ago by slow-freezing. Any clinic that offers donor embryo replacements from such programs are thus required to have a thorough understanding and technical training in thawing of slow-cooled embryos.

56.6 Open Versus Closed Systems

One issue that has received significant attention in the past few years is that of safety of cryopreservation for the recipient as well as the resulting babies born from these embryos. The most cumbersome is transmitted diseases, especially viruses. There are very strict regulations for egg, sperm, and embryo donations, and the donors are screened vigorously before being classified as eligible for donation. Furthermore, there have not been any reports of patients contracting diseases from frozen ETs. However, a growing concern is prompting development of closed systems for storage, where the embryos or cryomedia are never in direct contact with liquid nitrogen. This especially applies to vitrification techniques. Slow freezing is typically performed in sealed straws, ampoules, or cryovials, which all presumably prevent this exposure to potentially contaminated liquid nitrogen. Therefore, as long as the freezing carriers are used correctly, the risk of contamination of slow-frozen embryos remains dismissible. Thus, one of the most significant advantages of slow-freezing vessels is that they are completely sealed and, if used correctly, exclude any contact with liquid nitrogen (excluding what may condensate from air inside the vessel). The caveat of sealed straws and vials is that the larger volume does not allow vitrification with the current protocols and medium formulations, so slow freezing is the only reliable option when using these closed-system devices. Most vitrification systems, on the other hand, traditionally require placing the embryo on a carrier device, such as a thin membrane, with minimal amount of medium, followed by direct submersion in liquid nitrogen. These systems are highly criticized for exposing embryos to potentially contaminated fluids and storing them in communal dewars, where the risk of cross-contamination, albeit theoretical, is of concern. There are a few companies that are marketing closed-system vitrification equipment, but traditionally the higher cooling rate of these systems have been thought to yield inferior results compared to open vitrification systems.

56.7 Slow Freezing Versus Vitrification

Many factors are causing clinics to switch to vitrification instead of traditional slow freezing. Several studies suggest that vitrification results in significantly higher survival and developmental rates than slow freezing [19, 20, 68, 69]. As mentioned above, other studies failed to demonstrate a significant difference [17, 18]. A further driving force for the increased use of vitrification is the practicality of the technique. Apart from being much more cost-effective, the procedure requires significantly less time (typically about 20–30 min total) when compared to the long equilibration methods required by typical slow-freezing protocols (around 1.5–2 h). Thawing following these techniques also differs greatly, with warming a vitrified embryo taking about 15 min, versus approximately 45 min for slow-frozen embryos.

Despite these advantages of vitrification, some labs still use slow freezing, mainly because of the wealth of clinical data that supports the technique. Furthermore, recent reports on a modified, one-step slow-freezing protocol based on higher sucrose concentrations found no differences in thaw survival or subsequent outcomes between this altered slow-cooling and vitrification techniques, which significantly reduce the amount of time to complete the procedure. Paired with a less technically challenging method and a lower transportation risk that comes with larger volume vessels such as straws and vials, some labs may opt to keep using slow cooling as their primary choice for cryopreservation. Furthermore, switching a well-established, effective method for a new protocol seems intimidating, thereby preventing implementation of newer technologies in some more traditional labs. Although the number of labs that use vitrification has been steadily increasing, slow-cooled embryos are likely to be part of our field for the foreseeable future.

56.8 Legal and Ethical Considerations

The ability to store embryos for an indefinite amount of time brings up all sorts of ethical and legal issues. First and foremost, ownership of embryos has to be determined before patients that are interested in freezing are admitted to the treatment plan. This becomes very important when couples separate after freezing their embryos. It can also be an issue where family members may want ownership of leftover embryos. Patients should seek legal consultation to decide and clearly document what happens to their embryos in any of such circumstances.

Another troublesome issue for patients is that of discarding extra embryos. Often, patients are against destroying embryos but do not want any more children and also do not feel comfortable donating their embryos. These patients are left with no other option than to continue storing the embryos with the associated costs. Clinics may have difficulty collecting payments from patients after years of storage, and legally, clinics are not authorized to destroy embryos without patient consent. This can be a large burden on IVF programs, especially those with large frozen embryo collections, often from many years ago. The best way for clinics to prevent this situation is to give proper patient guidance on the subject and keep thorough records of patient consent forms before treatment begins.

56.9 Future of Slow Freezing in Cryopreservation

Embryo cryopreservation has become such an integral part of infertility treatment that it is hard to imagine that the first successful freezing and thawing of a human embryo was just over 30 years ago [16]. Slow freezing has undoubtedly played the most prominent role in embryo cryopreservation. However, because of discussed factors, vitrification is becoming the method of choice for more and more clinics, and it is only reasonable to expect that within the next decade, slow freezing for embryo preservation may be completely replaced by vitrification, despite several advantages of the technique as discussed above. Nonetheless, at the moment, many groups still rely on this much older technology for preserving embryos at all different preimplantation stages. Furthermore, slow freezing is routinely used for preservation of spermatozoa, somatic cell lines, and tissue biopsies. Therefore, although embryo cryopreservation may continue without this technology, the vast majority of clinical-freezing applications will continue to benefit from slow freezing.

Review Questions

1. What accidental discovery resulted in the first successful cryopreservation of sperm?
2. What is the most likely cause of cryodamage?
3. What are two different *types* of cryoprotectants and how does each of these prevent cryodamage?
4. What are the primary advantages and disadvantages of freezing embryos at 2PN, cleavage, and blastocyst stages, respectively?
5. How does embryo quality affect freeze/thaw survival?
6. What are the advantages and disadvantages of slow freezing vs vitrification?

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Vitrification of Embryos

Juergen Liebermann

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Learning Objectives

- To provide a brief overview of the principles, purposes, and goals of cryopreservation of embryos
- To discuss vitrification technique proving step-by-step protocols with special notes for the clinical embryologist
- To provide data supporting the successful application of embryo vitrification at the blastocyst stage

57.1 Brief Background

57.1.1 Principles and Purposes of Cryopreservation of Human Embryos

Since the announcement in 1972 of the survival of mouse embryos after cryopreservation at −196 °C [1], the impact of cryopreservation on the growth and improved efficiency of assisted reproduction in humans has become increasingly appreciated. Moreover, cryopreservation has also been shown to increase overall pregnancy rates while allowing for further selection of embryos. Indeed it is possible to achieve implantation and pregnancy rates with frozen-thawed embryos as high as those achieved with fresh embryos. Routine in vitro blastocyst culture and cryopreservation have been shown to increase pregnancy rates while allowing for better selection of embryos. With more reliable cryopreservation techniques, lower numbers of embryos are now being transferred, resulting in less high-order multiple pregnancies as well as increased healthy implantations.

The fundamental objectives for successful cryostorage of cells in liquid nitrogen at −196 °C can be summarized as follows:

1. Arresting the metabolism reversibly.
2. Maintaining structural and genetic integrity.
3. Achieving acceptable survival rates after thawing.
4. Maintenance of developmental competency post thaw.
5. The technique has to be reliable and repeatable.

57.1.2 The Vitrification Component

The major disadvantage of using cryostorage is that it can lead to crystallization of water and thereby can create new and unwanted physical and chemical events that may injure the cells that are being preserved. As early as 1985, ice-free cryopreservation of mouse embryos at −196 °C by vitrification was reported in an attempted alternative approach to cryostorage. Since then, vitrification techniques have entered more and more the mainstream of animal and human reproduction as an alternative cryopreservation method to traditional slow-cooling/rapid-thawing protocols. In addition, the last few years have seen a significant resurgence of interest in the potential benefits of vitrification protocols and techniques in human-assisted reproductive technologies. The radical strategy of vitrification results in the total elimination of ice crystal formation, both within the cells being vitrified (intracellular) and in the surrounding solution (extracellular). *Vitrification*, however, avoids ice formation altogether during the cooling process by establishing a glassy or vitreous state, wherein molecular translational motions are arrested without structural reorganization of the liquid in which the reproductive cells are suspended. The physical definition of vitrification is the solidification of a solution at low temperature, not by ice crystallization but by extreme elevation in viscosity during cooling [2, 3]. In general, vitrification solutions are aqueous cryoprotectant solutions that do not freeze when cooled at high rates to very low temperatures. In addition, the rate of cooling/warming and the concentration of the cryoprotectant required to achieve vitrification are inversely related. In addition, recent publications have shown the relatively greater importance of warming rate over cooling rates with regard to the survival of oocytes subjected to a vitrification procedure [4–6].

The earliest attempts using vitrification as an ice-free cryopreservation method for embryos were first reported in 1985 [7]. In 1993, successful vitrification of mouse embryos was demonstrated [8]. Furthermore, bovine oocytes and cleavage-stage embryos were vitrified and warmed successfully a few years later [9]. In 1999 and 2000, successful pregnancies and deliveries after vitrification and warming of human oocytes were reported [10, 11]. Since that time, and because it seems to be that both entities appear to be especially chill-sensitive cells in ART, blastocysts appear to have received a significant boost in survival rates by avoiding ice crystallization through use of vitrification [12].

Vitrification is very simple, requires no expensive programmable freezing equipment, and relies principally on the placement of the embryo in a very small volume of vitrification medium that must be cooled at extreme rates not obtainable in traditional enclosed cryostorage devices such as straws and vials.

It is important to mention that cryopreservation interferes without doubt with the intracellular and extracellular homeostasis and therefore poses a risk for inducing spindle abnormalities, chromatid nondisjunction, fertilization errors, and abnormal mitotic divisions during embryonic development. In addition, specific subcellular compartments containing accumulated transcripts and proteins necessary for spindle formation, epigenetic control, and chromosome alignment are also mandatory for proper spindle functionality. Although it is far too early to draw conclusions about the risk of imprinting mutations and birth defects after vitrification of oocytes and embryos, the current literature is reassuring. An increased incidence of imprinting diseases in live births after cryopreservation of oocytes and pre-implantation embryos has not been identified [13].

57.1.3 Cryoprotectant Agents

One "drawback" of vitrification considered by embryologists not familiar with the vitrification technique is the use of high concentrations of cryoprotectants, which does mean

that vitrification solutions are potentially more toxic than their counterpart solutions used for conventional slow freezing. This is necessitated by the practical limit for the rate of cooling and the biological limit of tolerance of the cells for the concentration of toxic cryoprotectants being used to achieve the cryopreserved state. It is important to note that recently published papers [14–17] have shown that the use of relatively high concentration of cryoprotectants, such as 15% (v/v) ethylene glycol (EG) used in an equimolar mixture with dimethyl sulfoxide (DMSO), had no negative effect on the perinatal outcomes from blastocyst transfers following vitrification when compared with those from fresh blastocyst transfers.

Cryoprotective agents (CPA) are essential for the cryopreservation of cells. Basically, two groups of cryoprotectants exist: (1) permeating (e.g., glycerol, EG, DMSO) and (2) non-permeating (e.g., disaccharides, proteins, and polymers) agents. The key component of a vitrification solution is the permeating agent. These compounds are hydrophilic non-electrolytes with a strong dehydrating effect. Furthermore, these CPAs are able to depress the "freezing point" of the solution. Additionally, these highly permeating cryoprotectants are also more likely to diffuse rapidly out of the cells, so that the cells quickly regain their original volume upon warming, thus preventing osmotic injury. The second component of a vitrification solution are the non-permeating CPAs such as disaccharides, for example, sucrose, which does not penetrate the cell membrane but helps to draw out more water from cells by osmosis and therefore lessen the exposure time of the cells to the toxic effects of the permeating CPASs. The non-permeating sucrose also acts as an osmotic buffer to reduce the osmotic shock that might otherwise result from the dilution of the cryoprotectant after cryostorage. Furthermore, permeating agents are able to bind with intracellular water, and therefore water is very slowly removed from the cell. During warming, using a high extracellular concentration of sucrose (e.g., 1.0 M) counterbalances the high concentration of the cryoprotectant agents in the cell, as it reduces the difference in osmolarity between the intra- and extracellular compartments. The high sucrose concentration cannot totally prevent the cell from swelling, but it can reduce the speed and magnitude of swelling [18–20].

57.1.4 Artificial Collapsing and Assisted Hatching

Most importantly, residual fluid in the blastocoel can reduce post-warming survival of embryos. Vanderzwalmen et al. [21] showed that survival rates in cryopreserved, expanded blastocysts could be improved by artificial reduction of the blastocoel cavity, and others consider that blastocoel collapse is necessary pre-vitrification on whichever day the blastocyst forms [21, 22] and the most effective strategy for promoting both permeation of cryoprotectant and dehydration [23].

Although one study has suggested that expanded blastocysts show no significant differences in viability, implantation potential, or pregnancy outcome when frozen on day 5 versus day 6 [24], our "body of data" [15–17] refutes the comparable implantation rates for blastocysts cryopreserved on day 5 or 6. And while there are several possibilities why day 6 blastocysts perform less well than day 5 blastocysts, one explanation could be that the older blastocysts are often more expanded with a significantly larger blastocoel impacting water loss and CPA absorption. Being mindful of previously published research on artificial collapse (AC) of blastocysts prior to cryopreservation, we looked for an opportunity that could potentially help us improve the outcome for day 6 blastocysts using AC of the blastocoel prior to vitrification. Because the concept showed improvement for the day 6 blastocysts, we applied to day 5 blastocysts that had well-expanded blastocoels.

AC of the blastocoel can be performed using different techniques, such as micro-needles, sucrose solutions, or lasers [25–30]. In 2003 and 2004, two groups independently reported a beneficial effect by applying AC to blastocysts prior to vitrification. Son et al. [25] observed an improved clinical pregnancy rate of 48% and an implantation rate of 29% with the use of AC. Hiraoka et al. [26] collapsed day 5 and day 6 blastocysts by manually pipetting embryos until they collapsed and achieved a clinical pregnancy rate of 50%, with an implantation rate of 33% after warming. Moreover, Mukaida et al. [27, 28] found that the survival rate of vitrified blastocysts was negatively correlated with the size of the blastocoel. They speculated that a large blastocoel may disturb the efficacy of vitrification. They collapsed the blastocoel by puncturing it with a micro-needle or by making a hole between two trophectoderm cells with a laser pulse. After applying AC, the survival improved from 86% to 97.2%. Moreover, their pregnancy rate went up from 34.1% to 60.2%, with an implantation rate of 46.7%. Iwayama et al. [29] used a laser pulse or osmotic shock resulting from exposure of the whole embryo to sucrose, and the implantation rate was significantly higher in both groups compared to the control group without AC (59.7% and 49.3% vs. 34.2%). Furthermore, Hur et al. [30] looked at the effect of AC, achieved using a 29 gauge needle or laser pulse, on clinical outcomes in fresh transfers, and they observed a significant increase in the clinical pregnancy in the study group compared to the control group (58.8% vs. 39.0%). All publications mentioned, including Liebermann and Conaghan [31] and Liebermann [32], conclude that AC has a beneficial effect both in frozen blastocyst transfers and for overall cumulative pregnancy and implantation rates. A recent study by Kazemi et al. [33] assessed the effect of pre-vitrification blastocoel fluid reduction on the survival, hatching rate, and the expression of genes related to apoptosis (*Tp53*), pluripotency (*Pou5f1*, *Nanog*), and differentiation (*Cdx2*, *Eomes*, *Gata6*) in mouse blastocysts. The survival rate of treatment groups was similar to the control group, whereas the hatching rate of artificial collapse/vitrified blastocysts was significantly higher than vitrified blastocysts.

Quantitative reverse-transcription PCR analysis revealed a considerable reduction in the expression of *Cdx2*, *Eomes*, *Gata6*, *Grb2*, and *Tp53* transcripts following artificial collapse/vitrification in comparison to the vitrification-alone group; the abundance of *Pou5f1* and *Nanog*, however, did not change. The authors concluded that artificial collapse of the blastocoel cavity before vitrification leads to relatively normal expression of apoptosis and development-related genes plus higher hatching rates.

Because of the post-thaw phenomena called "zona hardening" [34, 35] and based on recently published data [36], all thawed embryos underwent assisted hatching using a laser by removing about a third of the zona pellucida.

Warming of blastocysts occurred 1–2 h prior transfer. Both natural and hormone replacement cycles were used to increase the receptivity of the endometrium. Progesterone was supplemented on day 15 of the cycle, and blastocysts were warmed on day 6 of progesterone supplementation.

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57.2 Brief Overview of Method

57.2.1 Materials

1. HSV (High Security Vitrification Kit) (Catalog # 022137; Cryo Bio System)
2. Heat Sealer (Cryo Bio System)
3. Polycarbonate micropipettes, 275 μm end hole (MidAtlantic Diagnostics# KFPIP-1170-10BS)
4. Brady labeler (TS 2000)
5. Brady labels (PTL-19-427)
6. 90 × 15 mm petri dish (Nunclon # 150362)
7. Center-Well Organ Culture Dish (Falcon 3037)
8. Styrofoam container
9. Visotubes 10 mm (IMV 5561)
10. Cryo Canes aluminum (Thermo Scientific 5015–0001)

57.2.2 Reagents

1. Serum Substitute Supplement (SSS) (Irvine)
2. Wash solution as a part of the Thaw Kit (Irvine)
3. Vit Kit – Freeze (Irvine Scientific #90133DSOC)
4. Vit Kit – Thaw (Irvine Scientific # 90137DSOC)

57.2.3 Equipment

1. Dissecting stereo microscope (Olympus SZX-12, Bausch Lomb or Leica) with warming stage
2. Laminar flow hood (Origio)
3. Inverted microscope (Olympus IX-71)
4. Infrared 1.48 μm diode laser (Hamilton Thorne – LYKOs laser (Hamilton Thorne Research, Beverly, MA)

57.2.4 Methods

57.2.4.1 Stepwise Blastocyst Vitrification Procedure

Vitrification of blastocysts is undertaken utilizing a "closed system" (HSV, High Security Vitrification Kit; Cryo Bio System, L'Aigle, France; FDA 510(k) clearance for cleavage-stage embryos in blastocysts) (*see* Notes 1 and 2) after a two-step loading with cryoprotectant agents at 24 °C (*see* Notes 3 and 4). If assisted collapsing (AC) is done prior to vitrification, then the blastocyst is put on an inverted microscope equipped with a laser system (LYKOS, Hamilton Thorne), the junction of two trophectoderm cells in each blastocyst is located, and one shot (100% power, 500 μs pulse length) is applied to the cell junction. The blastocyst is then moved back in the incubator for 5–10 min. Briefly, blastocysts have to be placed in equilibration solution, which is the base medium (M199) with 20% Serum Supplement Substitution (SSS) containing 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethyl sulfoxide (DMSO) (see Table 57.1). After 8 min, the blastocysts need to be washed quickly in vitrification solution, which is the base medium containing 15% (v/v) DMSO, 15% (v/v) EG, and 0.5 M sucrose, for 60 s and transferred onto the HSV using a micropipette. Immediately after the loading of not more than two blastocysts in less than a 1 μL drop on the HSV, the straws are heat sealed, then plunged in LN2, and finally stored inside 5 mL liquid nitrogen prefilled canes (Visotube Rond, IMV, France). Each single step is described in detail below:

1. Aseptic techniques are required at all stages. For equilibration and vitrification procedures, ensure the benchwarmer is at room temperature (~24 °C) (*see* Notes 3 and 4).
2. Take reagents from the refrigerator and allow them to warm to room temperature.

Table 57.1 Summary of vitrification and warming solutions (Irvine Vit Kit "Freeze" and "Warm")

	Composition		
	Ethylene glycol (%)	DMSO (%)	Sucrose (M)
Equilibration solution (ES)	7.5	7.5	0
Vitrification solution (VS)	15	15	0.5
Warming solution (TS)	0	0	1.0
Diluent solution (DS)	0	0	0.5
Wash solution (WS)	0	0	0

M-199 + 20% SSS [wash solution, WS]

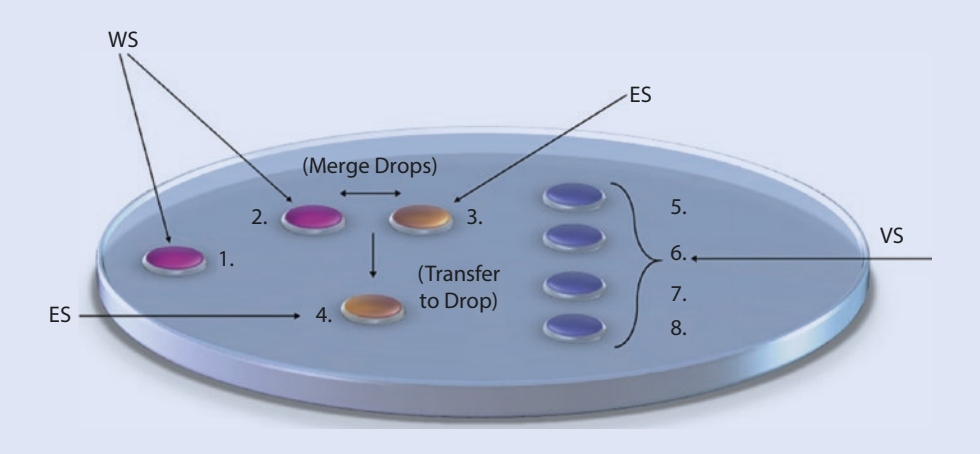

Fig. 57.1 Setup for the vitrification procedure on a plain 90 mm dish lid surface (ES, equilibration solution; VS, vitrification solution; WS, washing solution)

3. Move blastocysts to freeze into a separate well. Bring this dish to the inverted microscope, and with the embryo positioned with the laser objective, use a single pulse to hit the blastocysts between two trophectoderm cells to collapse the embryo. Place the dish back into the incubator for 5 min.
4. Label a petri dish with the patient's name under the lid as follows: washing solution (WS), equilibration solution (ES), and vitrification solution (VS). Prepare 2 × 50 µL of WS, 2 × 50 µL of ES, and 4 × 50 µL of VS (*see* Fig. 57.1).
5. Brady label should include the patients' last name, first name, accession number, medical personal identification [MPI] number, date plus number, and type of embryos.
6. Before vitrification, use a stripper tip with 275 µm end hole for loading the blastocysts on the top.
7. Fill Styrofoam container with LN_2.
8. Each sample that is vitrified will be done in a separate hood and verified by a second embryologist before proceeding. Vitrify good expanded/hatching blastocysts on days 5/6/7.
9. Remove embryos from culture dishes using a stripper tip into the WS (drop 1), gently aspirating to remove any residual culture medium.
10. Pipette from WS (drop 1) to the other drop of WS (drop 2), and immediately merge it with the first drop of ES (drop 3). Set the timer for 5 min.
11. After 5 min, transfer the embryos to the remaining drop of ES (drop 4). Set the timer for 3 min. Place the embryos on the top of the drop and let them settle to the bottom.
12. Then load the blastocysts in a VS back-loaded stripper tip, and rinse through the four droplets of VS (drop 5–8), and between each droplet, clean the tip.
13. Placement into the VS and loading of the HSV should take *less than 1 min*, so that the total incubation time in VS is 30 sec. After 30 sec, gently transfer them to the tip of the HSV by using a stripper tip to load the blastocyst(s) in as small volume (less than 0.5 µL) as possible onto the edge of the stick (*see* Note 5).
14. Visually confirm placement (*see* Note 6).
15. Before loading, apply the label to the open end of the empty straw. Load the HSV stick into the empty straw, the side with the embryos first. Use the blue handle to make sure the stick is in as far as it goes. Then, using the heat sealer, seal the open end of the stick, and plunge the whole straw into the LN_2. Place the straw in a pre-cooled aluminum cane for further storage (*see* Notes 7 and 8).
16. Store at the cane in a nitrogen tank.
17. Make sure to record cane location on the freezing worksheet and cryo inventory log.
18. Complete all paperwork, and recheck that all vial locations are logged into the Embryo Inventory.

57.2.4.2 Stepwise Blastocyst Warming Procedure

It is important to mention that regardless of the day of cryopreservation of the embryo (whether day 5, 6, or 7), at thawing, blastocysts should be treated as if they had been cryopreserved on the fifth day of development. To remove the cryoprotectants, blastocysts need to be warmed and diluted in a three-step process. With the HSV submerged in LN_2, the inner straw *should be* removed, and then the carrier with the blastocysts can be removed from the LN_2 and placed directly into a pre-warmed (37 °C) organ culture dish containing 1 mL of 1.0 M sucrose (*see* Note 9). Blastocysts can be picked up directly from the HSV and placed in a fresh drop of 1.0 M sucrose at 24 °C and immediately connected with a drop of 0.5 M sucrose for 3 min. Then connect with another drop of 0.5 M sucrose. After 2 min, blastocysts are transferred to a drop of pure 0.5 M sucrose solution and connected with drops of base medium for 3 min. Then connect with another drop of base medium for additional 2 min. When switching the embryos between different concentrations of warming

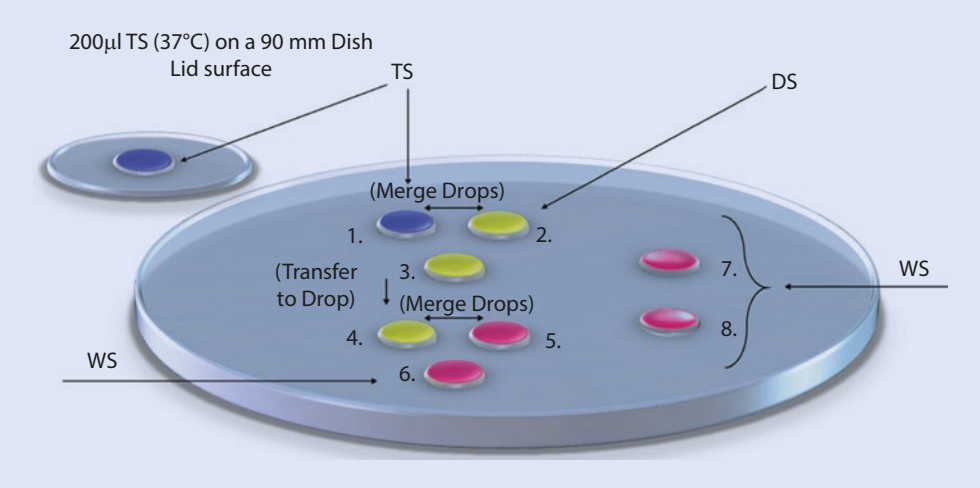

Fig. 57.2 Setup for the warming procedure on a plain 90 mm dish lid surface (TS, thawing solution; DS, diluent solution; WS, washing solution)

solutions, be sure to fill up the pipette with the next lower concentration of warming solution before picking up the cells for moving into the next dilution (*see* Note 10). Finally, the blastocysts are washed in the two drops of base medium for 1 min (at this moment, perform assisted hatching) and then returned to culture medium (SAGE+20%SSS) (SAGE blastocyst medium, Trumbull, CT, USA) in the incubator until transfer. Each single step is described in detail below:

1. Take reagents from the refrigerator and allow them to warm to room temperature. All cryoprotectants are removed at 24 °C.
2. Place a 200 µL drop of TS on a petri dish and place on a warming plate (*see* Note 9).
3. Label a petri dish (Nunc) with the patient's name under the lid as follows: TS, DS, and WS. Prepare 1 × 200 µL on a 90 mm dish and warm it up to 37 °C. Then prepare on a separate 90 mm dish lid 1 × 50 µL of TS, 3 × 50 µL of DS, and 4 × 50 µL of WS (*see* Fig. 57.2).
4. Before warming, use a stripper tip with 275 µm end hole for removing the blastocysts from the HSV tip.
5. Fill Styrofoam container with LN_2.
6. Confirm location and identification with a second embryologist before warming any HSV kit. Warm one kit at a time.
7. Each sample that is warmed is done in a separate hood and verified by a second embryologist before proceeding.
8. With the HSV kit under LN_2, open the kit by cutting the outer straw. Use the blue handle to remove the inner stick.
9. Submerge HSV kit directly in the pre-warmed 37 °C drop containing 200 TS, which should be as close as possible to the LN_2 Styrofoam container (*see* Note 11). As soon as the HSV kit contents liquefy (within 1 sec), try to locate the blastocyst(s) before removing it (them) with a stripper tip. After locating all the blastocysts, remove them from the HSV tip, and place them in the droplet of TS (drop 1) at room temperature, and then connect immediately with the first droplet of DS (drop 2). Wait for 3 min.
10. Then connect with the second droplet of DS (drop 3) for 2 min.
11. Then transfer blastocysts to 0.5 M sucrose (drop 4) and connect with first of WS (drop 5) for 3 min.
12. After 3 min, connect with second drop of WS (drop 6) for 2 min. Finally, rinse through a series of two additional drops of WS (drops 7 and 8).
13. Perform assisted hatching on the warming dish before placing the blastocysts into a culture dish, and put them back in the incubator for subsequent culture.
14. Record the survival and appearance of all blastocysts. Update log with warming data, and notify the physician of result (*see* Note 12).

57.3 Successful Application of Blastocyst Vitrification

Between 2004 and October 2016, the Fertility Centers of Illinois "IVF Laboratory River North "(Chicago) has vitrified 32,769 blastocysts from 8141 patients (Table 57.2). The majority of blastocysts were vitrified on day 5 (55.0%), 43.5% on day 6, with a minority on day 7 (1.5%). After 13 years of vitrifying blastocysts using an open (Cryotop) as well as closed (HSV) system, and close to 6700 FETs with an average number of 1.7 embryos transferred, the perinatal outcomes are as follows: number of babies from 1914 deliveries until January 2015 equals 2336 (1203 girls and 1133 boys) (Table 57.3). No abnormalities were recorded.

Table 57.2 Retrospective data from 8141 patients (average age 34.6 ± 6.0) with blastocyst cryopreservation by vitrification between 2004 and November 2016

	Day of development			
	Day 5	Day 6	Day 7	Total
Number of blastocysts vitrified	18,087 (55.0%)	14,211 (43.5%)	471 (1.5%)	32,769 (100%)

The outcomes with regard to day of development and age of the patient including donor-recipient cycles, between 2004 and October 2016, are summarized in Tables 57.4 and 57.5. In patients under 35 years old when transferring day 5 blastocysts, an ongoing pregnancy and implantation rate of 49.2% and 45.1% was noted (Table 57.4). In contrast, transferring day 6 blastocysts in patients younger than 35 of age, an ongoing pregnancy and implantation rate of 36.0% and 32.4% was recorded (Table 57.5).

In October 2007, the Fertility Centers of Illinois "IVF Laboratory River North" moved forward from the use of an open carrier system (Cryotop – embryos are in direct contact with liquid nitrogen) to a closed system (embryos are sealed before contact with liquid nitrogen). Using a closed carrier (High Security Vitrification Kit, HSV) for aseptic vitrification, the following data from day 5, day 6, and day 7 blastocysts were observed and are summarized in Table 57.6: (1) clinical pregnancy rate (cPR), 54.8% vs. 42.5% vs. 18.2%; (2) ongoing pregnancy (oPR), 46.3% vs. 33.3% vs. 15.9%; and (3) implantation rate (IR), 44.0% vs. 31.9% vs. 12.5% (Table 57.6). As shown in Table 6, oPR, cPR, and IR occurring in the day 5 blastocyst group were significantly higher than transferring day 6 or even day 7 blastocysts.

Between 2007 and October 2016, the Fertility Centers of Illinois "IVF Laboratory River North" (Chicago) performed 2765 vitrified blastocyst transfers (VBT) without collapsing prior to vitrification, with a mean patient age of 35.7 ± 4.9 years (group A), and in 2765 VBT (group B) with a mean patient age of 35.5 ± 4.8 years where artificial collapse was performed prior to vitrification (Table 57.7). On average, 1.6 vs. 1.5 embryos were transferred in group A vs. B, which means 40–50% of all VBT were single embryo transfers. Survival in group A versus group B was not significantly different (98.8% vs. 99.3%). However, there was a significant improvement in group B compared with group A for the following: (1) clinical pregnancy rate (cPR), 57.0% vs. 43.7%; (2) ongoing pregnancy rate (oPR), 48.5% vs. 34.7%; and (3) implantation rate (IR), 46.1% vs. 33.5% (Table 57.7).

When the vitrified-warmed blastocysts were divided into day 5 and day 6 groups, the following data were observed (Table 57.8): in 1622 VBT transferring day 5 blastocysts from group A (mean age of 35.5 ± 5.0), the IR, cPR, and oPR were 38.4%, 48.9%, and 40.0%, compared to 49.0%, 59.7%, and 51.5% of day 5 blastocysts from group B ($n = 1930$; mean age of 35.2 ± 4.8). As shown in Table 57.8, implantation, cPR, and oPR occurring from the day 5 blastocysts in group B were significantly higher than from the day 5 blastocyst in group A (χ^2; $P < 0.001$, respectively).

Table 57.3 Perinatal outcome of vitrified blastocysts after 6662 transfers between 2004 and October 2016 (babies delivered until January 2015). Values are numbers unless otherwise described; percentages are indicated between brackets

		Day of development		
	Day 5 + day 6	Day 5	Day 6	Day 7
Deliveries (total)	1914	1264	641	9
Babies born (total)	2336	1555	771	10
Female	1203	813	386	4
Male	1133	742	385	6
Singletons	1502 (78.5)	979 (77.5)	515 (80.0)	8 (89.0)
Twins	402 (21.0)	279 (22.0)	122 (19.0)	1 (11.0)
Triplets	10 (0.5)	6 (0.5)	4 (1.0)	–

Table 57.4 Retrospective outcome data (2004–2010/2016) at the *Fertility Centers of Illinois, Chicago*, from vitrified day 5 blastocysts in regard to the patient's age. Values are numbers, unless otherwise described

	Patient's age (y)				
	<35	35–37	38–40	>40	Donor
Average age	31.3 ± 2.4	35.9 ± 0.8	38.9 ± 0.8	42.6 ± 1.9	43.5 ± 4.8
Cycles	1978	895	587	307	355
Transfers	1977	894	585	306	355
Blastocysts survived	98.4	98.7	98.8	98.7	99.0
Blastocysts transferred (mean)	1.6	1.6	1.7	1.7	1.7
Positive pregnancy/VET (%)	64.9	62.9	60.5	59.8	62.8
Clinical pregnancy/VET (%)	56.0	52.0	49.0	48.0	53.0
Ongoing/delivered pregnancies (%)	49.2	44.0	39.0	37.0	41.0
Implantations	1442	585	355	173	240
Implantation rate (%)	45.1	42.0	36.5	34.3	40.5

Table 57.5 Retrospective outcome data (2004–2010/2016) at the *Fertility Centers of Illinois, Chicago*, from vitrified day 6 blastocysts in regard to the patient's age. Values are numbers, unless otherwise described

	Patient's age (y)				
	<35	35–37	38–40	>40	Donor
Average age	31.2 ± 2.3	36.0 ± 0.8	38.9 ± 0.8	42.6 ± 1.9	43.0 ± 4.7
Cycles	1039	579	451	316	178
Transfers	1032	574	448	314	177
Blastocysts survived	97.7	98.7	98.2	97.0	99.7
Blastocysts transferred (mean)	1.7	1.6	1.7	1.6	1.7
Positive pregnancy/VET (%)	52.6	48.4	48.0	45.5	46.3
Clinical pregnancy/VET (%)	44.0	41.0	40.0	35.0	38.0
Ongoing/delivered pregnancies (%)	36.0	33.0	31.0	24.0	27.0
Implantations	579	291	224	124	79
Implantation rate (%)	32.4	30.6	29.5	24.4	26.6

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Table 57.6 A comparison of retrospective data from the cryopreservation program (*Fertility Centers of Illinois, Chicago*) of vitrified day 5, day 6, and day 7 blastocysts using aseptic vitrification technology between October 2007 and October 2016. Values are numbers unless otherwise described; percentages are indicated between brackets

Day 5 + day 6 + day 7		Day 5	Day 6	Day 7
Patient's age (y)	35.7 ± 4.9	35.3 ± 4.9	36.2 ± 4.8	36.0 ± 4.1
Transfers	5575	3552	1979	44
Blastocysts warmed	9102	5679	3349	74
Blastocysts survived	9011 (99.0)	5635 (99.2)	3304 (98.7)	72 (97.3)
Blastocysts transferred	8856	5546	3238	72
Blastocysts transferred (mean)	1.6	1.6	1.6	1.6
Implantations	3483 (39.3)	2440 (44.0)[a]	1034 (31.9)[a]	9 (12.5)[a]
Positive pregnancy/VET	3334 (59.8)	2297 (64.7)[b]	1023 (51.7)[b]	14 (31.8)[b]
Clinical pregnancy/VET	2795 (50.1)	1947 (54.8)[c]	840 (42.5)[c]	8 (18.2)[c]
Ongoing/delivered pregnancies	2309 (41.4)	1643 (46.3)[d]	659 (33.3)[d]	7 (15.9)[d]
Live births (n) until 01/2015	1726	1200	520	6

[a,b,c,d] $P < 0.001$; *VET* vitrified embryo transfer

If we compare day 6 in group A ($n = 1143$; mean age of 36.1 ± 4.8) with day 6 outcome in group B ($n = 835$; mean age of 36.4 ± 4.7), the following data in terms of implantation, cPR, and oPR were observed: 26.6%, 36.4%, 27.3% vs. 39.7%, 50.7%, and 41.6%, respectively (Table 57.8). As shown in Table 8, implantation, cPR, and oPR occurring in the day 6 blastocysts of group B were significantly higher than transferring day 6 blastocysts from group A (χ^2; $P < 0.001$ for any comparison, respectively).

In Table 57.9, the results for patients under 35 years in groups A (no assisted collapsing) and B (assisted collapsing) are summarized. Comparing day 5 from group A ($n = 760$; mean age 31.4 ± 2.3) with day 5 from group B ($n = 950$; mean age 31.4 ± 2.3), we found the following for IR, cPR, and oPR: 41.5% vs. 53.0%, 52.0% vs. 62.7%, and 45.0% vs. 55.3%. Looking at day 6 outcomes for group A versus group B, we observed the following for IR, cPR, and oPR: 30.2% vs. 44.1%, 40.6% vs. 55.8%, and 32.9% vs. 46.5% (see Table 57.9).

Table 57.7 A comparison of retrospective data from the cryopreservation program (*Fertility Centers of Illinois, Chicago*) of vitrified day 5 and day 6 blastocysts without artificial collapsing (group A) and with artificial collapsing day 5 and day 6 blastocysts (group B) using aseptic vitrification technology between 2007 and October 2016. VET = vitrified embryo transfer. Values are numbers unless otherwise described; percentages are indicated between brackets

	Technique	
	Group A	Group B
Patient's age (y)	35.7 ± 4.9	35.5 ± 4.8
Transfers	2765	2765
Blastocysts warmed	4739	4286
Blastocysts survived	4681 (98.8)	4255 (99.3)
Blastocysts transferred	4562	4216
Blastocysts transferred (mean)	1.6	1.5
Implantations	1526 (33.5)[a]	1945 (46.1)[a]
Positive pregnancy/VET	1423 (51.5)[b]	1895 (68.5)[b]
Clinical pregnancy/VET	1209 (43.7)[c]	1576 (57.0)[c]
Ongoing/delivered pregnancies	960 (34.7)[d]	1341 (48.5)[d]

[a]$P < 0.01$; [b,c,d]$P < 0.001$

57.4 Conclusions and Future Directions

Vitrification is a very promising cryopreservation method with many advantages and an ever increasingly consistent clinical track record. A standardized vitrification protocol applicable to all stages of the pre-implantation embryo may not be realistic because of (1) different surface-to-volume ratios; (2) the pattern of the movement of water and cell-permeating cryoprotectants that is rather more stage specific; (3) differing cooling rate requirements between oocytes, zygotes, cleavage-stage embryos, and blastocysts; and (4) variable chill sensitivity between these different developmental stages. Currently, however, the most widely used protocol applied to any embryo stage is the two-step equilibration in an equimolar combination of the cryoprotectants ethylene glycol and DMSO, at a concentration of 15% each (v/v) supplemented with 0.5 mol/l sucrose. For the adoption of vitrification in ART, as with all new technologies, there has been initial resistance; but as clinical data has been accrued, this technology is becoming more commonly adopted as standard procedure in many IVF programs worldwide. With this increased use in human-assisted reproduction will come evolution of the vitrification process as it is fine-tuned to clinical needs so pushing forward its development to higher levels of clinical efficiency, utilization, and universal acceptance.

Table 57.8 A comparison of retrospective data from the cryopreservation program (*Fertility Centers of Illinois, Chicago*) of vitrified day 5 and day 6 blastocysts without artificial collapsing (group A) and with artificial collapsing (group B) using aseptic vitrification technology between 2007 and October 2016. VET = vitrified embryo transfer. Values are numbers unless otherwise described; percentages are indicated between brackets

	Technique			
	Group A		Group B	
	Day 5	Day 6	Day 5	Day 6
Patient's age (y)	35.5 ± 5.0	36.1 ± 4.8	35.2 ± 4.8	36.4 ± 4.7
Transfers	1622	1143	1930	835
Blastocysts warmed	2735	2004	2943	1343
Blastocysts survived	2709 (99.0)	1972 (98.4)	2925 (99.4)	1330 (99.0)
Blastocysts transferred	2632	1930	2913	1306
Blastocysts transferred (mean)	1.6	1.7	1.5	1.6
Implantations	1012 (38.4)[a]	514 (26.6)[aa]	1426 (49.0)[a]	519 (39.7)[aa]
Positive pregnancy/VET	926 (57.1)[b]	497 (43.5)[bb]	1370 (71.0)[b]	525 (62.9)[b]
Clinical pregnancy/VET	793 (48.9)[c]	416 (36.4)[cc]	1153 (59.7)[c]	423 (50.7)[cc]
Ongoing/delivered pregnancies	648 (40.0)[d]	312 (27.3)[dd]	994 (51.5)[d]	347 (41.6)[dd]

Day 5, [a]$P < 0.01$; [b,c,d]$P < 0.001$
Day 6, [aa]$P < 0.01$; [bb,cc,dd]$P < 0.001$

Table 57.9 A comparison of retrospective data from the cryopreservation program (*Fertility Centers of Illinois, Chicago*) of vitrified day 5 and day 6 blastocysts without artificial collapsing (group A) and with artificial collapsing (group B) using aseptic vitrification technology in patients younger than 35 years old between 2007 and October 2016. Values are numbers unless otherwise described; percentages are indicated between brackets

	Technique			
	Group A		Group B	
	Less than 35 years old		Less than 35 years old	
	Day 5	Day 6	Day 5	Day 6
Patient's age (y)	31.4 ± 2.3	31.6 ± 2.2	31.4 ± 2.3	31.4 ± 2.4
Transfers	760	468	950	301
Blastocysts warmed	1299	839	1418	485
Blastocysts survived	1281 (98.6)	820 (97.7)	1404 (99.0)	483 (99.6)
Blastocysts transferred	1246	799	1404	478
Blastocysts transferred (mean)	1.6	1.7	1.5	1.6
Implantations	517 (41.5)[a]	241 (30.2)[aa]	744 (53.0)[a]	211 (44.1)[aa]
Positive pregnancy/VET	443 (58.3)[b]	222 (47.4)[bb]	695 (73.1)[b]	209 (69.5)[bb]
Clinical pregnancy/VET	395 (52.0)[c]	190 (40.6)[cc]	596 (62.7)[c]	168 (55.8)[cc]
Ongoing/delivered pregnancies	342 (45.0)[d]	154 (32.9)[dd]	525 (55.3)[d]	140 (46.5)[dd]

Day 5, [a, b, c, d] $P < 0.001$
Day 6, [aa, bb, cc, dd] $P < 0.001$; *VET* vitrified embryo transfer

57.5 Practical Implications Vitrifying at the Blastocyst Stage

Our data have shown that freezing at the blastocyst stage provides excellent survival, implantation, and clinical pregnancy rates. To achieve these outcomes, consider these points: (1) without a successful blastocyst vitrification storage program, extended culture should never be attempted; (2) the blastocyst is composed of more cells and is therefore better able to compensate for cryoinjury; and (3) the cells are smaller, which makes cryoprotectant penetration faster. On average, fewer embryos per patient are cryostored, but each one has a greater potential for implantation when thawed.

57.6 Addendum: Special Notes for the Clinical Embryologist

1. Special care must be given to the selection of the vitrification carrier type. It is necessary to use types of carrier or vessel material with rapid heat transfer that also support the process of uniform heat exchange to achieve higher cooling rates.
2. In addition, although no reports of contamination in human IVF following cryopreservation exist, the user should be encouraged to choose a closed carrier system, which in our experience works for blastocysts without any problems.
3. To minimize the toxicity of the cryoprotectant, a stepwise exposure of cells to precooled concentrated solutions (approximate room temperature of 24 °C) is recommended.
4. Utilizing higher concentrations of cryoprotectant allows shorter exposure times to the cryoprotectant – but be careful: the potential toxicity of the cryoprotectant increases at higher concentrations. As almost all cryoprotectants are toxic to some extent, it is important to carefully monitor the duration of exposure to the final cryoprotectant before plunging into liquid nitrogen.
5. To facilitate vitrification by higher cooling rates, it is also necessary to minimize the volume of the vitrification solution (VS) as much as practical (preferably less than 1 μL). From this point of view, it is very important to use a small pulled pipette. Furthermore, by collecting the blastocysts in one place and loading no more than two blastocysts at the same time in the pipette, it is possible to keep the volume small. However, if the load of media is too large, it can still be reduced before plunging in LN_2 by "drawing down" the droplet to flatten the blastocysts slightly while removing all surplus vitrification solution.
6. To make sure that the blastocysts are loaded on the carrier, perform the loading process under a stereomicroscope. Always confirm the number of loaded blastocysts.

7. After sealing the carrier, submerge the carrier loaded with the blastocysts directly in liquid nitrogen by passing rapidly through the vapor phase (nitrogen gas).
8. Store the cryo-cane in a pre-chilled PVC cryo-sleeve sitting in the goblet in the dewar. It is essential to maintain exposure of the HSV to LN_2 at all times to eliminate risk of warming and de-vitrification.
9. Before moving the carrier quickly from the liquid nitrogen into the warming solution, have a stripper tip (micro-pipette) ready. Fill the pipette with a small amount of the first warming solution (TS). When using the HSV as the vitrification carrier, rinse the open edge of the straw after placing into the pre-warmed (37 °C) 1.0 M sucrose; because the droplet is so small, it warms immediately, and it is essential to pull/stir the blastocysts off the carrier surface as soon as possible to avoid any toxic effect of the VS. A stirring motion is recommended when plunging into the warm TS to agitate the cell off the carrier surface without the need to remove it actively.
10. When switching the cells between different concentrations of warming solutions, fill up the pipette with the next lower concentration of warming solution, before picking up the blastocysts to move into the next concentration (dilution effect).
11. In general, during vitrification and warming, the LN_2 Styrofoam box needs to be as close as possible to the working area to minimize any lag in cooling and warming rates.
12. Be aware of the expiration dates of the vitrification and warming media; once opened, the shelf life is 6 (six) weeks (according to recommendation of Irvine Scientific).

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Review Questions

1. Please summarize the five fundamental objectives for successful cryopreservation of cells.
2. What has the most impact on the successful vitrification/warming of eggs and embryos: cooling or warming rates or both?
3. What would be a major disadvantage of cryopreservation if not done properly?
4. What is the most important characteristic of vitrification, if performed properly?
5. Name the two different groups of cryoprotectant, and provide the name of two cryoprotective agents for each group.
6. What role does sucrose play during the vitrification as well as warming procedure?
7. At what temperature should vitrification of eggs or blastocysts be performed?
8. Describe two procedures that might support survival and successful implantation of blastocysts: one being applied before vitrification of blastocysts, and one after warming before transfer.

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Ovarian Tissue Cryopreservation

Pasquale Patrizio and Sherman Silber

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Learning Objectives

- Limitations of conventional freezing of oocytes and embryos for fertility preservation.
- Ovarian tissue freezing and re-transplantation should become routine procedures.
- How to perform ovarian tissue harvesting and where to re-transplant.
- Methods to evaluate ovarian tissue for presence of cancer cells.
- Assessment of return of ovarian function and the hormonal testing.
- The difficulty of whole ovary transplant.
- Update on the number of births after ovarian tissue cryopreservation.

58.1 Introduction

As cancer survival rates keep improving, women of reproductive age need options to preserve their future fertility from the gonadotoxic doses of chemotherapy and/or radiotherapy [1–4]. Freezing embryos and oocytes are now established methods of fertility preservation. Embryo freezing has a long track record of success, and women can expect excellent pregnancy rates from cryopreserved embryos, with success rates in the United States ranging from 19% to 36% depending on a woman's age at the time of embryo freezing [5, 6]. However, the disadvantage of this method is that a woman needs a husband, or male partner, or donated sperm. For women wishing to preserve fertility but without a male partner or not interested in donor sperm, oocyte cryopreservation is a viable option. Pregnancy rates from oocytes cryopreserved by vitrification have been approaching that seen from cryopreserved embryos in some centers, and it is no longer considered experimental [7–9]. However, freezing oocytes requires a delay in the cancer treatment, and generally only one cycle of ovarian stimulation can be offered.

This chapter will focus on the cryopreservation and re-transplantation of ovarian tissue [10–12]. While this method of fertility preservation is still considered experimental as opposed to embryo or oocyte freezing, it does offer important advantages, and, in our opinion, as well as those of others [13], it should no longer be considered as "experimental." Contrary to embryo or oocyte cryopreservation, which requires approximately 10 days of ovarian stimulation, potentially resulting in high serum estradiol levels, ovarian tissue can be harvested and cryopreserved on short notice. It can be offered to women who need an almost immediate start chemotherapy or who have an estrogen-responsive cancer such as some types of breast cancer. It can also be offered to pre-pubertal girls, who are increasingly surviving from childhood malignancies, yet they may lose their fertility as a result of chemotherapy [14–17]. Indeed, ovarian tissue cryopreservation may be the only viable option for children with malignancies who need to undergo gonadotoxic treatment and wish to preserve future fertility. Finally, there will be a brief review of the experience with whole ovary cryopreservation, keeping in mind, however, that whole ovary is nowadays no longer an advocated method for fertility preservation, being replaced by the freezing of ovarian cortical tissue.

58.2 Cryopreservation of Ovarian Cortical Strips

Ovarian tissue for cryopreservation can be extracted either by laparoscopy or by minilaparotomy, and both these types of surgery can be performed at any point in a woman's menstrual cycle [10, 18]. The ovarian cortex harvested during laparoscopy or minilaparotomy is cut thin in 1 mm strips, either after removing approximately half the ovary in a block of cortical tissue or by taking small 5 mm biopsies. A different approach is to remove one ovary (unilateral oophorectomy) and then excise cortex "on the bench" in the lab. The use of cautery must be avoided during ovarian cortex harvesting. Due to the unpredictable assessment of the risk for chemo-/radiotherapy-induced gonadotoxicity, some women can be expected to have some ovarian function remaining or returning after chemo or radiotherapy; therefore, it is prudent to leave behind one ovary. With an experienced surgeon, the median operating time is 30 min. The complications from harvesting ovarian cortical strips or performing unilateral oophorectomy, via laparoscopy or minilaparotomy, are minimal and not different from any other ovarian surgery.

The ovarian cortex contains a multitude of primordial and immature follicles, and cryopreservation of the ovarian cortex is able to safeguard these large numbers of follicles to be used at a later time. The primordial and immature follicles are undifferentiated and not metabolically active [19, 20]. The small water content, the high surface-to-volume ratio, and the absence of the zona pellucida may mean that primordial follicles are less susceptible to damage during the process of cryopreservation. However, as the ovarian cortical strips are frozen without their vascular supply, they rely on neovascularization for their post transplant survival. Depletion of follicles in fact may occur when the ovarian graft is reimplanted until a new vascular supply (generally after 3 days to a week) is established to perfuse the graft [21–24]. Slow freeze has been shown to result in a 60% loss of oocytes compared to vitrification, which results in reduced oocyte loss. But oocyte loss may not matter clinically, because there are hundreds of thousands of oocytes in just one ovarian cortex [25].

Successful fresh as well as frozen ovarian cortex transplants in humans were first published in 2004 and 2005 as case reports, and many other case reports have subsequently followed [10, 11, 26–30]. There is now a rapidly growing interest in cryopreservation of ovarian cortical tissue prior to sterilizing cancer treatment followed years later by re-transplantation after cancer has been cured. It is estimated that some 100 births have been achieved and reported in either official publications or personal communications. The possibility is also looming of preserving fertility and even hormonal function against the natural decline caused by

aging and to delay menopause [31–33]. Furthermore, ovarian tissue cryopreservation may also find future indication to allow healthy young women to electively preserve their future fertility instead of using oocyte freezing. However, the main benefit of ovarian tissue over oocyte cryopreservation remains for cancer patients who need to start their chemo-/radiotherapy promptly and cannot afford to wait around for two or three cycles of IVF to accumulate and bank sufficient number of oocytes and knowing that the pregnancy rate per single oocyte is only about 4–5% [34]. With ovarian tissue freezing and subsequent re-transplantation, they can potentially get pregnant naturally and preserve several hundred thousand oocytes.

The first human applications of ovarian tissue freezing and re-transplantation were preceded by a long history of animal experimentation. As far back as 1954, Deanesly showed in rats and, in 1960, Parrott showed in mice that ovarian tissue could be successfully frozen and autografted resulting in live births [35, 36]. Interest in human applications began after Gosden's report of successful pregnancies in sheep in 1994 [19]. Recently, one of the most interesting case reports in humans involved rejuvenating menopausal ovarian cortex with cryopreserved autotransplantation [37]. Interest in cryopreserved ovarian cortical transplantation is rapidly growing, although only two reports to date have clearly stated its success rate [38, 39]. No systematic report had been published from one center comparing fresh donor transplants and cryopreserved ovary autografts, and little had been gleaned from studies of these procedures on analysis of ovarian function and resting follicle recruitment prior to a recent publication [39]. A recent worldwide survey of 37 babies born from cryopreserved transplants still could not establish a clear success rate [32]. However, a summary of cases from a single series of both fresh and cryopreserved transplants from one center, carried out with the same technique and assessed uniformly over follow-up, with the aim of improving our understanding of resting follicle recruitment, demonstrated the clinical robustness of the procedure [30, 39]. All of the recipients had normal return of hormonal and menstrual ovarian function at about the same time after surgery (4.5 months).

The duration of function of *fresh* grafts was directly related to the original ovarian reserve of the donor. In all cases, only one-quarter to one-half of the donor ovary was transplanted and most of the tissue cryopreserved for future use. All grafts functioned for more than 2 years, over one-half of them for over 6 years, and two of them already for over 8 years. Thus, if the donor's ovarian reserve is high, these grafts can last for a long time despite reduced AMH levels [25, 31]. That has very important implications for understanding the mystery of primordial follicle recruitment.

The relationships among FSH levels, menstruation, and AMH levels in donor and recipient in fresh transplants are indicative of resting follicle recruitment and ovarian reserve [40]. As recipient FSH levels returned to normal within 130–170 days, the low AMH level of recipients then began to rise in response to an increasing number of mature gonadotropin-sensitive follicles [39]. The AMH of recipients continued to rise to well above the normal baseline AMH level of the donor. In the fresh allograft recipient shown, although FSH decreased to normal levels by day 133 and normal menstrual cycling resumed, AMH levels rose far above normal (higher than the donor) shortly thereafter. Despite the transplanted graft continuing function, AMH then returned to low levels. This analysis of both donor and recipient data after transfer is rare and useful in its demonstration of no significant long-term change in donor AMH levels despite loss of an ovary. The rise of recipient AMH levels above donor AMH levels reflects over-recruitment of resting follicles in the recipient compared with the donor. Contrary to what might have been expected based on earlier studies [21], no evidence was found of significant loss of follicles from transplantation ischemia, as each patient had FSH levels return to normal as AMH levels rose far above normal. Despite continued function of the transplanted graft, AMH then returns to low levels [30, 39]. Preservation of follicles was supported by our observation of substantial follicle recruitment with many eggs produced in the two patients who underwent IVF during the window of high AMH levels. Quantitative studies have also supported this finding in bovines [41].

The autotransplantation of *frozen* ovary tissue yielded results almost identical to fresh transplantation. As with fresh ovary transplants, FSH levels returned to normal by about 150 days in all cases, and menstrual cycles resumed shortly before that. The return to normal did not differ between slow freeze cases and vitrification cases. In all of the cryopreserved cases, just as with fresh transplants, AMH rose to high levels shortly after FSH returned to normal, at around 130–170 days. Then, exactly as with fresh transplant cases, AMH dropped to a lower baseline level by about 240 days and remained at that lower level [12, 30]. Eight of the eleven cryopreserved autotransplant recipients had a follow-up of over 1 year, which allowed the assessment of pregnancy potential. Seven of these eight patients spontaneously conceived, although one spontaneously aborted. The other six were healthy singleton pregnancies. In fact, there are now even 3 more healthy babies from 11 frozen transplants followed for over a year. Therefore, cryopreserved and fresh transplantation were similar in hormonal function and high pregnancy outcome. Functional hormonal results thus far have demonstrated a remarkable degree of repeatable concordance in all 22 cases of fresh ovary donor transplantation and cryopreserved ovary autotransplantation. Thus, the spontaneous pregnancy rate overall reached 75% [39].

58.2.1 Orthotopic or Heterotopic Re-transplants?

In humans, cryopreservation of ovarian cortical strips as a method of fertility preservation has emerged over the last 15 years [12, 30, 39] and ovarian cortical strips have been autotransplanted either into the pelvis (orthotopic) or outside the pelvis (heterotopic) [10, 12, 26–29, 42–44]. Orthotopic

autotransplantation involves transplanting the thawed ovarian cortex onto the remaining ovary or into a peritoneal window in the pelvis. The transplantation surgery can be done laparoscopically or microsurgically through minilaparotomy. The first pregnancy and live birth from fresh ovarian cortical tissue was achieved in 2004 after orthotopic transplantation [10]. A 25-year-old with stage IV Hodgkin's lymphoma underwent a laparoscopy to biopsy the cortex of the left ovary. Five biopsies were taken and then cryopreserved. The patient received chemotherapy and radiation and then became amenorrheic. Five years later, the ovarian cortex was thawed and then transplanted into a peritoneal window that had been created 7 days earlier to help promote angiogenesis. Eleven months after reimplantation of the ovarian cortex, an intrauterine pregnancy was documented, and the patient went on to deliver a healthy son.

When thawed ovarian cortex is transplanted onto a remaining ovary or on nearby peritoneum, there is not always need for follicular aspiration and assisted reproductive techniques, as the fallopian tube can pick up and transport the spontaneously ovulated oocyte. However, if the ovarian cortex is transplanted elsewhere, then follicular aspiration and in vitro fertilization are required. Initially, controversy existed because it could not be proven in the cases of orthotopic transplantation that the live birth was not a result of residual ovarian function from the remaining ovary. However, this initial skepticism is subsiding as so many of live births from the procedure have been reported.

Overall, at least 100 live births have been described after orthotopic transplantation of frozen-thawed ovarian cortex ([30, 39] and personal communications), and some were after spontaneous conception and others after in vitro fertilization but all except one from orthotopic re-transplants.

Heterotopic transplantation of ovarian cortex tissue to other sites such as the forearm or subcutaneous abdominal tissues has been described [44, 45]. Only one successful heterotopic transplantation has led to the birth of a healthy baby after oocyte production, fertilization, and transfer [45]. As live births after heterotopic ovarian cortex transplantation are extremely rare, this approach to ovarian tissue reimplant is nowadays rarely recommended.

58.3 Risks

One major concern of cryopreserving tissue from patients with a malignancy, and later reimplanting this tissue, is the risk of ovarian metastasis from the primary cancer [46]. Although thus far there have been no cases of transmission of cancer cells from transplanting back-thawed ovarian tissue, there is potential concern with leukemia [47, 48]. However, even with leukemia, if the ovary is removed and the cortex frozen, while the patient is in remission, there will be no viable cancer cells in the tissue [39]. In contrast, in a Danish study, in which females with leukemia had ovarian cortex cryopreservation, six of eight patients had PCR evidence of leukemic cells in the ovarian tissue [47]. Molecular markers of malignant cells allowed detection of small numbers of cells by PCR which could not be detected by histologic examination [49].

Another group examined ovarian cortex biopsies of a patient with CML and did not find evidence of malignant cells by routine histologic methods, but again, identified some evidence of malignant cells by PCR. It is unclear whether this PCR evidence of malignant cells came from the ovarian biopsies or from contaminating blood [46]. The viability of these malignant cells is unknown.

Advanced-stage breast cancer (stages III and IV) can metastasize to the ovary, and in patients with known advanced metastatic breast cancer, it may be prudent not to cryopreserve and then reimplant cortical strips. In contrast, more than ten women with Hodgkin's lymphoma have received reimplanted ovarian cortex tissue, and no relapses have been documented so far [50–52]. At any rate, ovarian cortex tissue should always be examined for malignant cells, and as new molecular technologies to assess minimal residual disease become available, they should be used to assess the ovarian tissue prior to being reimplanted.

Of note, one of the authors (S.S.) has been freezing ovarian tissue since 1996 for 100 young women with solid organ cancer, or with a great risk for POF, of whom 16 had spare frozen tissue subjected to detailed viability testing before cryopreservation and after thawing. All had histologic review by a variety of pathologists. Only one had ovarian metastasis, a young woman with widespread breast cancer metastasis throughout her entire body. Otherwise, none of the other cases had any tumor cells in their ovary. Andersen et al. have also noted a complete lack of ovarian metastasis, even in the majority of leukemia cases. The reason for the remarkable absence of ovarian metastasis might possibly be due to the fibrous avascular nature of the ovarian cortex [52, 53]. In fact, the reason why fetal ovarian tubules (which in the fetal male become seminiferous tubules) invade the fibrous cortex and become follicles is that the dense fibrous tissue of the cortex (which in the fetal and adult testis is just tunica albuginea) is needed to suppress the resting follicles from developing all at once prematurely [53].

58.4 Whole Ovary

Cryopreservation of ovarian cortical strips, thawing, and then reimplantation leads to some follicular loss secondary to ischemia; however, this does not affect successful results. An alternative to cryopreserving ovarian tissue has been the cryopreservation and transplantation of a whole human ovary. In theory, by re-establishing a quick vascular supply, there should be less follicular loss. However, many challenges had to be overcome including optimizing methods for cryopreservation of large organs such as the ovary, which can be difficult as cryoprotectants do not diffuse well into whole organs and intravascular ice formation can cause vascular injury. Also, the technical aspect of harvesting and reimplanting a whole ovary requires a skilled surgeon to preserve a long and intact vascular pedicle at the time of harvesting

and then again a skilled microsurgeon to perform the vascular reanastomosis at the time of reimplantation.

Whole ovary autotransplantation was first described using fresh ovaries in various animal models, including rats, sheep, and monkeys. The first and only successful whole ovary transplant in humans was reported by Silber et al. by using a fresh whole ovary [54].

The preceding work in animal models involved sheep ovaries, which were autotransplanted into the abdominal wall with microsurgical vascular anastomosis of the ovarian to the inferior epigastric vessels. After 7 days, the ovaries were removed and noted to have surviving follicles [55].

In a rodent model, successful transplantation of ovaries, fallopian tubes, and the upper segment of the uterus en bloc after cryopreservation was reported in 2002. In four out of seven attempts at transplantation, the rat ovaries survived 60 days or more, and one pregnancy was achieved [56]. Recently, adult female sheep have become a standard model to study intact ovary cryopreservation [57, 58]. Arav et al. described transplantation of frozen-thawed intact ovaries in eight sheep by artery and vein anastomosis to the contralateral ovarian artery and vein. From 24 to 36 months after the ovary was reimplanted, progesterone activity was detected in three sheep. Oocyte retrieval was successful in two sheep, and in embryonic development, up to the 8-cell stage was noted [59]. Bedaiwy et al. described restoration of ovarian function in frozen-thawed sheep ovaries reimplanted with microvascular anastomosis. Yet, 8 of 11 ovaries failed due to thrombosis at the pedicle site [60]. In 2006, a live-born lamb was reported after orthotopic microvascular reanastomosis of a whole cryopreserved ovary [61]. Whole ovary cryopreservation in the sheep has been attempted through both slow-cooling and vitrification methodologies [58, 59, 62–64]. Recently, ovarian function of cryopreserved and transplanted whole sheep ovaries has been demonstrated 6 years after transplantation. This is the longest reported ovarian function of frozen-thawed whole ovaries [65].

In humans, the first report of cryopreservation and then thawing of a whole ovary was described by Martinez-Madrid et al. in 2004 [66]. They found that the percentage of live follicles was 99.4% in fresh tissue, 98.1% after cryoprotectant exposure, and 75.1% after thawing, and they also reported high survival rates of stromal cells and small vessels after thawing [66]. Bedaiwy et al. have recently described that successful cryopreservation of the human ovary in two premenopausal women with overall viability of the primordial follicles was 75% and 78% in intact cryopreserved-thawed ovaries [60]. Further, Patrizio et al. reported on successful whole human ovary cryopreservation with the vascular pedicle utilizing a Multi-Thermal-Gradient device and a slow-cooling, rapid-thawing protocol (Fig. 58.1) [67, 68]. The ovaries were thawed after cryopreservation for 2–4 days, and the frozen-thawed ovary was histologically similar to the fresh contralateral ovary used as a control and modest increase in markers of apoptosis. In three cases, the fallopian tube was cryopreserved along with the whole ovary, and after thawing, the histologic architecture was intact [69].

Fig. 58.1 Seeding of whole ovary in the glass tube prior to being loaded in the Multi-Thermal-Gradient device for cryopreservation

Table 58.1 A comparison of cryopreservation of cortical strips vs. whole ovaries

	Cortical strip	Whole ovary
Laparoscopic harvesting possible	Yes	Yes
Preserves ovarian stroma	No	Yes
Loss of follicles after cryopreservation	Yes	Yes
Loss of follicles due to ischemia	Yes	Yes
Microvascular anastomosis possible[a]	No	Yes
Short-term endocrine function	Yes	Yes
Long-term endocrine function	No	Yes

Adapted from Bromer and Patrizio [68], with permission
[a]In the event of anastomosis failure, the whole ovary will be lost. Cortical strips offer an advantage here because multiple strips are harvested and transplanted. If the loss of one cortical strip occurs, the others may remain viable

At the time of this writing, however, no cases of reimplanting a frozen-thawed whole ovary resulting in a live birth have been reported in humans. It is possible that the risks of whole ovary transplantation are greater than with ovarian cortical strips, as transplantation of a whole ovary may result in a higher risk of metastasis. Also, when transplanting organs such as a whole ovary, if the vascular anastomosis fails, then the whole ovary is lost. This compares to the transplantation of cortical strips, which can be reimplanted in batches so that if the initial procedure fails, it can be repeated with the remaining cortical strips. See Table 58.1 for a comparison of the cryopreservation of whole ovaries versus cortical strips.

58.5 Conclusions

There is a large need for fertility preservation options for cancer survivors, and the time has come to consider ovarian tissue cryopreservation for fertility preservation no longer an experimental option. Ovarian cortex banking and re-transplantation has been shown to be successful with a large number of live births even after many years after cancer has been cured. Further research to perfect methods of cryopreservation of ovarian tissue should aim at enhancing follicular survival after thawing and to discover methods to activate complete folliculogenesis in vitro on the cortical strips. Concerning the whole ovary, at this time, there is interest in researching methods to carry out in vitro perfusion of the organ and induce folliculogenesis in vitro, but not for re-transplanting whole ovaries [70]. The current outlook is hopeful that soon ovarian tissue cryopreservation will be an accepted, established treatment option for fertility preservation.

Review Questions

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1. What are the limitations of conventional freezing of oocytes and embryos for fertility preservation?
2. Why should ovarian tissue freezing and re-transplantation become routine procedures?
3. How to perform ovarian tissue harvesting and where to re-transplant?
4. What are the methods to evaluate ovarian tissue for presence of cancer cells?
5. How to assess the return of ovarian function after re-transplant?
6. What are the difficulties of whole ovary transplant?
7. Have live births been documented after ovarian tissue re-transplantation?

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Establishing and Managing Donor Oocyte Banking

Kathryn J. Go, Paula Dwan, and Linda Hillis

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Learning Objectives

- Array of resources required to establish a donor oocyte bank
- Strategy for oocyte donor recruitment and compensation
- Processes for screening and acceptance of prospective oocyte donors
- Validated and effective clinical and scientific standard operating procedures for donor ovarian stimulation and method for oocyte cryopreservation (vitrification), respectively
- Federal regulations regarding donor reproductive cells and tissue
- Utility and advantages of donor oocytes in family building for some patients
- Development of marketing of inventory to recipients

59.1 Introduction

For almost four decades following the birth of the first IVF baby in 1978 [1], in vitro fertilization (IVF) and the array of assisted reproductive technologies (ART) have made it possible for countless individuals to realize the dream of having a family. Remarkable technical advances in IVF were rapidly made to enhance fertilization and optimize embryo implantation and utilization. The catalogue of ART evolved to include freezing (cryopreservation) of cleavage-stage embryos [2] as well as zygotes (fertilized eggs) and blastocysts, assisted hatching [3], intracytoplasmic sperm injection (ICSI) [4], and embryo biopsy for preimplantation genetic testing for single-gene mutations and/or chromosome copy number (aneuploidy screening) [5, 6]. What eluded inclusion in this repertoire for many years was the ability to cryopreserve eggs despite an early report of a live birth [7].

Multiple advantages are afforded by egg freezing. In providing an alternative to freezing *embryos* in a treatment cycle, the patient who elects to freeze her supernumerary eggs and her partner can avoid the ethical dilemma of the disposition of potential "surplus" embryos, i.e., more embryos than are needed to build their family. In instances when sperm are not available on the day of retrieval, the eggs can be frozen, deferring the insemination. Egg cryopreservation, importantly, offers the prospect of fertility preservation to reproductively aging women as a strategy to manage ovarian loss or function from gonadotoxic treatments such as surgery or chemo- or radiotherapy and potential loss or injury during military service or from an accident or against declining ovarian reserve with age.

A significant contribution to ART is the success of egg freezing in fulfilling an obvious need in third-party reproduction, or family building requiring donor gametes and gestational carriers. While the availability of frozen donor sperm through commercial banks or private clinics had been well-established for treatment with home insemination, or intra-uterine insemination (IUI) or ART, infertility therapies requiring donor eggs were realized with the advent of IVF and the use of eggs from "fresh" donation, i.e., ovulation induction of and egg retrieval from the egg donor [8]. Treatment was comprised of the selection of an egg donor from those available through specialized agencies or clinics by the recipient, the egg donor's ovarian stimulation and egg retrieval in a treatment cycle hormonally synchronized with the egg recipient's, and, finally, transfer of the resultant embryo(s) to the recipient's uterus with optimal endometrial preparation.

Fresh egg donation offered excellent prospects for clinical pregnancy and live birth rates for recipients as reflected in the annual infertility clinic reports of the Society of Assisted Reproductive Technology (► https://www.sartcorsonline.com) but entailed a high cost per treatment cycle or attempt (typical total fees for one attempt can be approximately $35,000), the contracting with an anonymous donor through an agency or clinic, and coordination of the donor's stimulation and monitoring of hormonal and ovarian follicular response to achieve synchronization with the recipient's. Given the significant commitments of time and effort by the donor to the cycle, the recipient was necessarily dependent on her availability to undergo the stimulation and retrieval, introducing inconvenience and potentially, a protracted waiting time. Cost-efficiency and convenience were not hallmarks of this treatment modality: there was a clear need to have donor egg availability achieve better access.

Effective egg freezing opened the door to egg "banking." The advantages are multiple: (1) with the advantage of distributing eggs from a single retrieval cycle among multiple recipients, cost-efficiency for each donor stimulation is achieved; (2) the temporal burden of donor and recipient cycle synchronization is eliminated, offering significantly greater convenience; and (3) greater access afforded by frozen egg banks increased the opportunity to many more patients whose reproductive treatments required donor eggs [9–13].

The availability of a reliable egg cryopreservation method readily fulfilled two clinical needs: the ability of reproductively aged women to preserve their fertility and the creation of donor egg banks analogous to sperm banks. The latter was an obvious opportunity to provide a significantly more convenient and affordable option and greater access to individuals desiring donor eggs for family building compared to identifying a suitable donor whose stimulation and retrieval cycle was dependent on her availability as well as possible synchronization with the recipient's cycle to afford optimal timing for embryo transfer. While egg freezing and storage for a clinic's patient for her own use (autologous egg freezing) follow the same model as that applied for cryopreserved embryos created through a treatment cycle, commercial *donor* egg banking entails more resources.

The establishment and management of a cryopreserved donor egg bank are multifactorial processes and require an array of expertise and operations. The establishment must include (1) a laboratory that has acquired, validated, and implemented the laboratory methodologies of egg vitrification and inventory management, is registered with and accredited and licensed by all appropriate regulatory agencies, and has an electronic database that captures and tracks

outcomes for each donor's eggs from number of eggs acquired to number of live births to achieve quality assurance and control; (2) a systematic approach for (a) recruiting, screening, and accepting appropriate egg donors; (b) the medical and clinical expertise for appropriately and thoroughly evaluating the donor candidates; and (c) managing the donors from ovulation induction cycle through egg retrieval; (3) an organizational plan that provides (a) an administrator and manager who oversee the development of the bank and its catalogue by building and nurturing relationships with its donors and clients and (b) a business director responsible for the financial and fiduciary management of the bank and oversight of its contracts and agreements with partners; and (4) a multidisciplinary leadership team attuned and attentive to the bank's continued development and quality.

This chapter provides a framework for establishing and managing a donor egg bank by addressing each of these elements, presented in ◘ Table 59.1.

59.2 The Science, the Technology, and Laboratory Requirements

Identifying a consistent and reliable method for freezing eggs, unlike sperm, was a challenge. Although a report of a pregnancy from thawed eggs frozen by slow cooling appeared in 1986 [7], the method could not be immediately replicated.

The slow-cooling method that proved successful for the freezing of zygotes (di-pronucleated fertilized eggs), embryos, and blastocysts involved equilibration of the samples with cryoprotectants and cooling in programmed freezing machines to achieve gradual reduction in temperature, e.g., rates such as 0.3° per min. Thawing or warming was achieved with a brief exposure to room temperature followed by equilibration in thawing or warming solutions to achieve stepwise rehydration of the zygote, embryo, or blastocyst.

Encouraging outcomes from egg freezing by slow cooling were reported [14–16], but it was the application of the ultrarapid freezing achieved by vitrification that catalyzed the emergence of a reproducible methodology for freezing and warming eggs with acceptably high recovery, survival, and potential for fertilization and embryo development.

Vitrification, in contrast to slow cooling, entails the equilibration of the egg with much higher concentrations of cryoprotectants followed by immediate immersion (plunging) of the sample into liquid nitrogen. The effective freezing rate with vitrification is the ultrarapid −20,000° per min, producing a glassy or vitreous intracellular environment and avoiding all ice crystal formation and its attendant damage to the cell [17, 18]. This technique was successful in addressing the unique challenges posed by eggs owing to their large diameter, high water content, and intracellular architecture, i.e., the meiotic spindle, requiring protection against disruption and the result risk of aneuploidy [19–22].

Warmed eggs do require fertilization by ICSI [23], reflecting potential low-temperature-induced changes to the zona pellucida precluding fertilization by conventional insemination.

Meticulous technique in vitrification must also be applied to achieve the desired post-warming survival, fertilization, and developmental potential for effective egg banking. Cryopreservation-related damage can result from nonadherence to the technical protocol that is specific in duration of equilibration intervals in cryoprotectant solutions and the loading of the eggs onto the carriers (the plastic appliances holding the vitrified eggs for storage). In addition, appropriate handling of the carriers must be achieved during transfer from storage vessel to vessel, e.g., storage tank to shipper, and shipper to storage tank. Best practices will prevent unintended warming of the eggs that will negatively affect their integrity, survival after warming, and ability to be fertilized and develop into embryos. Effective and validated training in conjunction with a solid foundation in reproductive biology and the principles of cryobiology will foster the quality assurance and quality control that contribute to the success of the bank. Thus, the prospective laboratory of the egg bank must ensure mastery of the technology with a structured training plan documenting practice by and validation of each scientist who will participate in egg retrieval, evaluation of maturity, and vitrification of mature (Metaphase II) eggs for addition

◘ **Table 59.1** Organizational and checklist chart for establishment and management of an egg bank

Scientific/laboratory	Medical/clinical	Administrative/business
Effective and validated egg freezing methodology Training for quality and consistency Appropriately equipped laboratory Storage facilities System for distribution Licensure and accreditation Registration with FDA Quality assurance/quality control programs	Compliance with ASRM guidelines Medical, psychological, and social history criteria for acceptance as an egg donor Application database Donor candidate evaluation and vetting (genetic screening, ovarian reserve screening) Risk factor assessment, infectious disease testing, physical examination Achieving safety for the egg donor and recipient	Egg bank's mission and values Development of contracts and business agreements Financial management of bank Provision of guarantees to recipients Development of business for growth and enhanced services to recipients/clients

to the bank's inventory. These professional scientists are likely to be clinical embryologists, well-versed in collecting eggs from ovarian follicular fluid, enzymatically removing the cumulus cells surrounding the eggs and evaluating the eggs for nuclear maturity. The mature eggs, that is, eggs at Metaphase II, are those that will be frozen.

With the burgeoning of favorable clinical experience and reports of pregnancies and live births from vitrified eggs in the medical literature, the American Society for Reproductive Medicine lifted the qualifier, "experimental," from egg vitrification in 2013 [24]. Subsequent reports reflect that egg freezing by vitrification has become firmly incorporated among the ART available to patients [25, 26] and has been deployed to commercial as well as autologous egg banking.

59.2.1 Storage and Distribution

An egg bank must have adequate storage capacity and meticulous maintenance of the storage containers, a redundant system for inventory management, e.g., both electronic and hardcopy versions, and a system for tracking inventory and accounting for the disposition of all samples – eggs that are in storage, shipped to other sites, or removed and discarded.

A database containing details of egg warming, specifically number of eggs surviving thaw and undergoing ICSI and number fertilized; number of embryos or blastocysts that developed and were transferred, cryopreserved, or discarded; and the clinical outcomes of any transfers, whether fresh or from a frozen embryo transfer (FET) cycle, must be in place for quality assurance and control. These data are central in the management and development of the bank's inventory, administering any guarantee programs and rendering decisions on the recycling of the egg donors, or conversely, the discontinuation or withdrawal of donors.

For shipment of eggs to other centers, dry shippers that have been validated through a program documenting that appropriate internal temperature is maintained over multiple days are used. If recipient centers send their tanks to the egg bank for loading of the samples, then the bank must inspect these to ensure they are fully functional. The use of colorimetric markers to reflect maintenance of the internal temperature over time is an excellent tool for routine use during shipping.

59.3 Compliance with Regulatory Agencies

The US federal government is clear in its rules for bank or establishment registration with the Food and Drug Administration (FDA) and the procurement, processing, storage, and distribution of donor cells and tissues: these are provided in the Federal Register, 21 CFR Part 1271 [27]. Donor eggs are considered and regulated as reproductive HCT/P – human cells, tissues, and tissue-based products. The ultimate objective of the regulations is to prevent the transmission of infectious disease by treatments using the cells or tissue. Fulfillment of these rules is achieved by the infectious screening of blood samples drawn from the candidate donor and the medical review of the outcomes that underpins whether an individual is eligible or ineligible as a donor.

The donor egg bank must register each year as an HCT/P-producing center, describing each type of product from the bank, and is subject to unannounced inspections by FDA officials. Included in the inspection are a survey of all the documents, procedures, and policies of the bank, interviews with its personnel, inspection of its facilities, and review of all donor records. Penalties for failures in compliance can be severe, with the possibility of closing of the bank's operations.

Complementing the federal regulations are the requirements by individual states that the egg bank must apply for a license as a tissue bank. Each state's application will vary in the breadth of information that must be provided to qualify for initial and renewed licensure.

While an egg bank could have an independent laboratory dedicated solely to egg retrieval, processing, vitrification, and storage, it is more frequently the case that the bank exists within and is part of an IVF clinic. While the egg donors can undergo ovulation induction and cycle monitoring at remote physician's offices or other centers, egg retrieval will occur at the bank where the eggs can be vitrified in its laboratory. ART laboratories for egg banks must have licenses as tissue banks, be registered with the FDA, and be certified as compliant with CLIA 88, the Clinical Laboratory Improvement Amendment of 1988, as well as the American Association of Tissue Banks (AATB), depending on state-specific requirements. As well, the laboratory is likely to be a member of the Society for the Assisted Reproductive Technologies (SART) of the American Society for Reproductive Medicine (ASRM).

59.4 Donor Recruitment, Evaluation, and Selection

In addition to the laboratory component, a donor egg bank requires both administrative and clinical staff working in conjunction toward bringing in young, healthy egg donors for ovarian stimulation. Administrative personnel and nurses work together to advertise to and recruit donors, review donor applications for clinical contraindications, accept applications contingent upon donors passing screening tests, coordinate screening tests, and ultimately stimulate the egg donor to produce a large cohort of oocytes.

A key component in establishing and maintaining a donor oocyte bank is the recruitment and screening of egg donors [28–30]. This is best accomplished through online advertising, social media, and local recruitment efforts. Knowing the target audience is crucial. The ideal age of donors is 21–30 years of age. Recruitment strategies must be focused toward healthy, young women, with a sense of altruism who would benefit from the compensation. They must be healthy, motivated, compliant, eager, and, ideally, educated. Initial online screening should be focused on the following areas: age, body mass index, health status, family history, and level of education. If the prospective donors do not meet

these basic criteria, it is not worthwhile to expend additional energy. If the prospective donors do meet these criteria, a much more extensive online application is the next step. Donors must submit demographic information, specific personal and relationship information, lifestyle information, detailed medical and surgical history for the donor, detailed medical history for first and second degree family members, as well as subjective information such as favorite subjects in school, hobbies and interests, and a personal statement about why they are interested in egg donation.

Donors may be disqualified at this second level of screening for poor lifestyle choices, personal or family history of autoimmune disorders, cancers at young age, or any other host of medical or social concerns. It is important to remember the attributes that donor recipients may be looking for when they undertake the donor selection process. If a recipient is not likely to choose a donor, then it does not make sense to continue further testing for that prospective donor.

Once the donor application has been vetted, it is then imperative for the nurse(s) to coordinate and perform ovarian reserve testing, genetic screening, psychological screening, infectious disease screening, risk factor screening, and physical examination including a detailed list of the donor's piercings and tattoos. Ideal egg donors will have a high normal ovarian reserve, not be carriers of common or high impact genetic diseases, be psychologically sound, be free of infectious diseases, and possess no limiting risk factors. All rules and regulations with respect to tissue banking must be adhered to in accordance with FDA policies.

Donor screening must be stringent. It is estimated that only 1–2% of women applying to be egg donors will actually meet all of the criteria set forth. Once the donors have been vetted, it is necessary to provide appropriate, detailed information about the egg donation process, including risk and benefits, time commitment, and compensation. All egg donors will need to complete informed consent. All prospective donors must be thoroughly educated about how to administer medications and subcutaneous injections, expected side effects, expected schedule for testing by blood draw and ultrasound, as well as the egg retrieval procedure and related anesthesia or sedation used to perform the egg retrieval.

It is the role of the nurse to provide the majority of the education regarding the treatment plan and medications used to stimulate the donor's ovaries. Ovarian stimulation protocols may vary, but must take into account the age and relatively high ovarian reserve potential of donors. Also, it must be remembered that these women are not considered infertile. As is the case in most IVF treatment cycles, the nurse(s) will provide overall education and day-to-day contact with the egg donor for medication instructions as the ovarian stimulation progresses. Detailed written medication and injection instructions are strongly recommended and will allow the donor to follow along and reduce medication errors on the part of the donor. Many donors will appreciate receiving their daily instructions via email, rather than verbally. Most donors have email accounts on their cellular phones and prefer this method of communication. Although most stimulation medications are now given by subcutaneous injections, minimizing the number of injections and length of time the donor will be subjected to medications and injections is imperative. The nurse will also provide preoperative instructions prior to the egg retrieval and postoperative follow-up after the egg retrieval. Nurses must ensure that donors feel well-cared for throughout the process if they hope to retain donors for subsequent donation cycles. Meeting with the donor on the day of the egg retrieval or soon after, thanking her for the donation and gift, and providing her with the compensation are strongly recommended.

According to guidelines set forth by the American Society for Reproductive Medicine, donors may participate in up to a maximum of six donation cycles [31]. It is financially beneficial for donor egg banks to utilize donors multiple times to offset the time and financial costs inherent in repeated screening.

59.5 Development of the Egg Bank and Future Prospects

The successful development of a donor egg bank must include the continual replenishment and expansion of the catalogue to offer potential recipients a greater array of donor phenotypes. As some ethnicities and mixed-ethnicities may be underrepresented among donors, an effort to recruit among these groups may fill potential voids in availability.

Every clinical and scientific technique should be subject to constant effort to achieve improvement, measured in terms of efficiency and/or efficacy. The safety of the donor during ovulation induction can be realized from innovations in controlled hyper-stimulation. With respect to the freezing method, constant revisions in cryoprotectant formulations or the technical steps of processing the eggs to achieve even higher survival, fertilization, and embryo development rates could lead to a reduction in the number of eggs needed to comprise a "lot" (the number of eggs purchased for a single treatment) consistent with leading to embryo transfer and live birth and potential supernumerary embryos for cryopreservation and future FET cycles. Potential improvements in design or function of cryopreservation storage tanks or shipping containers would also be advantageous to an egg bank for efficient operation.

59.6 Conclusion

The advent of effective egg freezing through vitrification with high survival and developmental potential offered significant advantages to individuals seeking fertility preservation as well as an improved model for patients using donor eggs. Advantages of lower cost, higher convenience, and greater access compared to the established model of fresh donor eggs were realized. The establishment and effective management of a donor egg bank is the result of careful planning and organization to ensure solid technology underpinned by committed quality assurance and control, a program of medical, genetic,

and psychological screening that is both regulation compliant and dedicated to the safety and protection of both donor and recipient, and a responsible and accountable administrative and business infrastructure for optimal donor and consumer relationships and financial management. A committed and dedicated team comprised of business, administrative, clinical/medical, laboratory, and marketing personnel is central to achieving success in the area of donor egg banking.

Review Questions

1. List the components that comprise a donor oocyte bank.
2. Describe the strategy for recruiting oocyte donor candidates.
3. Outline the processes for screening and acceptance of prospective oocyte donors.
4. Describe the clinical and scientific methods involved in creating an inventory of donor oocytes.
5. Identify the federal legislation that covers donor reproductive cells and tissues and summarize how a donor egg bank complies with this law.
6. Outline the advantages of cryopreserved donor eggs to a recipient.
7. Propose a viable marketing plan for the donor oocyte bank.

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Embryo Transfer and Advanced Treatment Options

Contents

Single Embryo Transfer

Thorir Hardarson and Matts Wikland

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Learning Objectives

- The advantages and disadvantages with SET
- The ideal patient for SET
- Indications for DET

Assisted reproduction technology (ART) is the single most effective method to treat infertility [1]. This high success rate has generally been achieved through replacement of multiple embryos resulting in a high multiple births rate, being 25% of all ART live births in the USA in 2014 [2, 3]. In the USA, the *ASRM practice committee* [4] recommended that between one and five embryos be replaced depending on the maternal age, number of IVF cycle, quality of the embryo, and/or the prognosis. It has been advocated that it should be up to the infertile couple and their physician to decide how many embryos should be transferred. In contrast, other countries like Sweden, Finland, Belgium, and Turkey have promoted a stricter line in the form of legislation/recommendations restricting the use of multiple embryo replacement. In those countries, the trend has been steadily toward reducing multiple pregnancies through the use of single embryo transfer (SET). Globally, however, there are large variations between how SET has been implemented, and even though countries like the USA have moved from 1% (2004) to 21% (2014) [3, 5], the rate of twin gestation has plateaued and 36% of all twins and 77% of higher-order multiples are still due to ART [6]. The annual expenditures for these iatrogenic preterm deliveries total 26 billion dollars of healthcare costs in the USA [7–9]. A situation likely to persist unless there are major changes in the way ART is viewed, funded, and legislated.

60

60.1 Multiple Pregnancies

The most important reason for decreasing the number of replaced embryos is the need to decrease the high incidence of multiple gestation and multiple birth rates (MBRs) produced through ART. In the USA, it has been estimated although ART only accounts for 1% of all births, ART births are 18% of all multiple births [10]. Despite that the majority of children born after multiple pregnancies are healthy, there are significant problems linked to multiple births both obstetrical and neonatal [11]. The obstetrical risks include hypertension, preeclampsia, preterm labor, anemia, and an increased Cesarean section rate. The neonatal risks include increased mortality, lower gestational age, low birth weight, and respiratory distress syndrome along with numerous other complications associated with preterm birth. In addition, long-term neurological complications have been associated with preterm birth along with the strain such a birth puts on the family both psychologically and financially.

Multiple births are associated with increased costs for the society compared to singletons. It has been estimated that the total cost of ART-associated preterm deliveries in the USA is approximately one billion dollars annually [2]. In the Netherlands, it has been estimated that lifetime extra healthcare costs of a twin pregnancy add up to €30,000 [12].

What can be done to reduce MBR in relation to IVF? Embryo reduction has been used to escape the complications associated with higher order of multiple pregnancies. Although embryo reduction is a relatively safe method, it however is not a solution for the quantitatively largest problem of twins, which are normally not reduced to singletons.

The only realistic way of avoiding multiple pregnancies in IVF is to reduce the number of embryos replaced. The problem with this approach is that in order to really reduce the multiple pregnancies, only one embryo should be transferred. By doing so, the total delivery rate will be affected negatively [13]. The question is then what is an acceptable delivery rate per transfer or started cycle? The attitude among patients as well doctors working with IVF is that the delivery rate should be as high as possible. With such an attitude, more than one embryo should be transferred. However, growing evidence indicates that by doing so, the problem with the high MBR in IVF will never be solved.

60.2 Pregnancy Results After SET

Pregnancy rates calculated per transfer are understandably the golden standard on success as it relates to an output parameter that is easily understood, gives fast feedback to the clinic on its performance, and relates the fresh cycle most commonly performed in IVF. There are of course many ways of comparing results and success. One other way of determining success is to calculate the cumulative birth rate per oocyte retrieval. Such an approach decreases the emphasis on the fresh transfer adding the increasingly important factor of cryopreservation of human embryos.

60.3 Results from Studies Comparing SET and Double Embryo Transfer (DET)

Comparing pregnancy results of SET contra multiple replacements has been reported in numerous randomized prospective trials [14–20]. The RCT studies show a clear advantage of transferring two embryos as compared to one. DET produces a significantly higher ongoing clinical pregnancy rate (44.5%) than SET (28.3%) [13].

Several studies show, however, that comparable ongoing clinical pregnancy rates can be achieved by transferring two embryos, one at a time [17, 18, 21]. However, such an approach requires a very good cryopreservation program. Improved techniques for cryopreservation have dramatically increased both the survival rates and the ongoing pregnancy rates.

Generally, RCTs are very valuable in clinical research, as their study design results in a similar distribution of confounding variables over the study arms. However, the normal IVF clinic cannot be a never-ending RCT study; it is a mixture of many different embryo transfer regimes that have been established over time. Those cohort studies that have been published reflect this reality [22–25]. They have

compared SET vs. DET and show no significant difference in the ongoing pregnancy rate, while there is a 30-fold decrease in the twinning rate in the SET group.

60.4 The Swedish Experience of SET

Due to the high MBR, resulting in criticism from obstetricians and neonatologists, Swedish IVF clinics already in 1993 on their own initiative started to reduce the number of transferred embryos from three to two. This resulted, as expected, in a dramatic drop in triplets, whereas the twinning rate remained unchanged at around 20%. Due to the known less favorable outcome for twins as compared to singletons shown in large follow-up studies of all IVF children born in Sweden between 1982 and 1995 [26], voices were raised that only one embryo should be transferred in the majority of cycles. Due to the debate concerning the high MBR clearly related to the number of transferred embryos and unchanged twinning rate, a multicenter randomized controlled trial was set up in Scandinavia by a group in Sweden [17]. The aim of the study was to compare one fresh embryo and, if no live birth occurred, adding another embryo transfer from the frozen thawed embryo, with transfer of two fresh embryos. The results did not show any difference in pregnancy rates between the groups with one fresh plus one frozen/thawed, as compared to the DET group. However, the twinning rate was 0.8% and 33%, respectively [17]. Without waiting for the final result from the above-mentioned study, the Swedish IVF legislation was revised with regard to the number of embryos that could be transferred. Thus, the Swedish National Board of Health and Welfare in their new guidelines in 2003 stated that only one embryo should be replaced in the majority of cases. However, the guidelines did not state in which situation only one embryo was allowed to replace. A detailed guideline was left to the profession to outline. The Swedish IVF clinics today, thus, recommend that only one embryo shall be transferred in the first two cycles in women under the age of 38 years. With this recommendation, almost 70% of the transfers performed in Sweden between 2003 and 2008 utilized only one embryo, and according to the latest Swedish IVF statistical report presenting delivery data between 2000 and 2015, the SET rate is 80% in fresh cycles (Fig. 60.1). During the same period, the overall delivery rate per transfer has remained fairly stable. For frozen/thawed cycles, the SET rate is 96% for 2015 (Fig. 60.2). The MBR has dropped to around 5%. Data from our own clinic also show that the overall pregnancy results are not affected by increasing the proportion of SET below 10% in 1998 to 86% in 2010 (Fig. 60.3). It is also interesting to notice that in women 40 years or younger, the birth rate remained stable, while a dramatic decrease in MBR occurred [27]. Comparing the Swedish

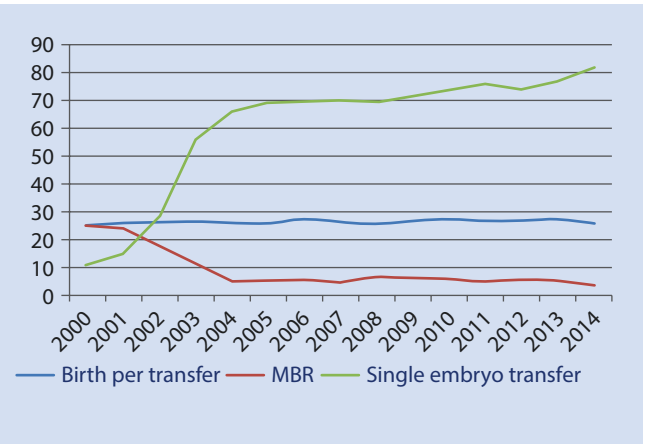

Fig. 60.1 Birth per transfer (standard IVF + ICSI), multiple birth rate (MBR), and single embryo transfer in Sweden in 2000–2015

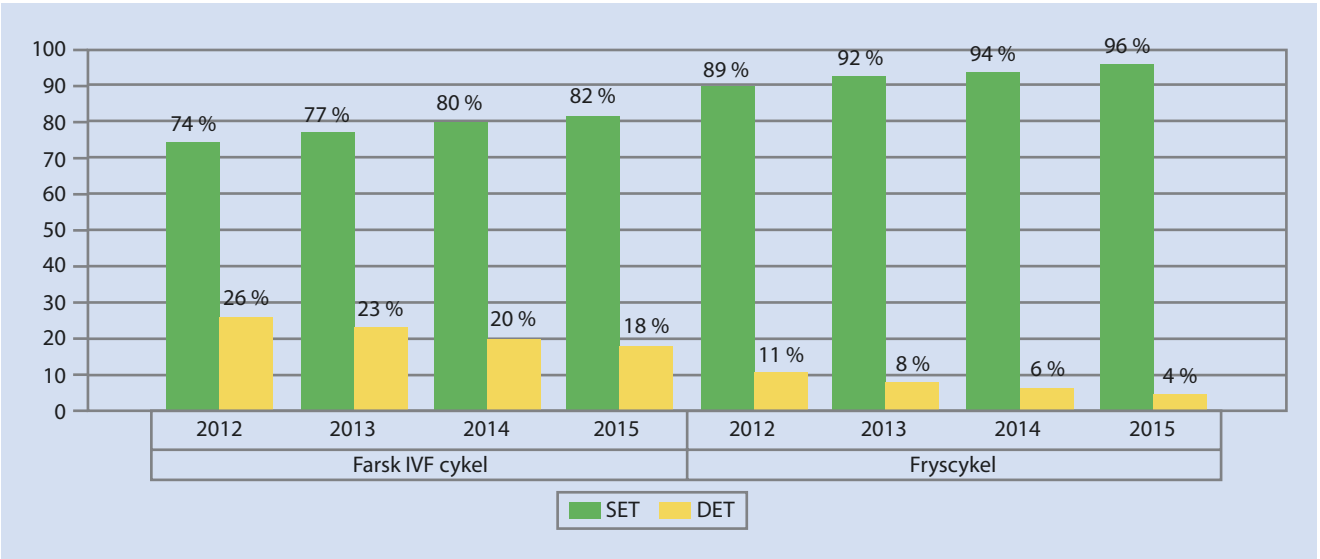

Fig. 60.2 SET and DET rate of fresh and frozen/thawed cycles in Sweden 2012–2015

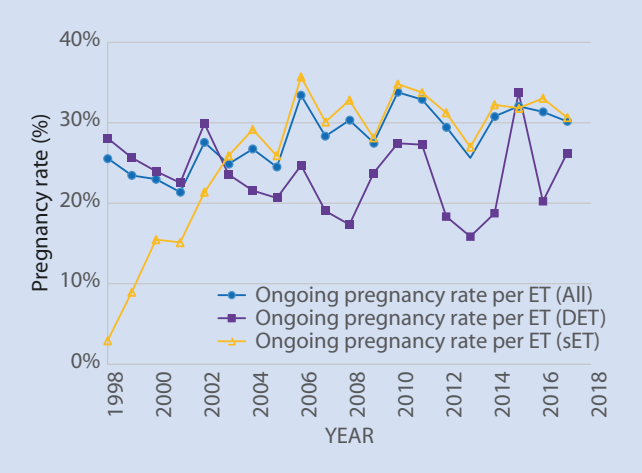

Fig. 60.3 Ongoing pregnancy rate after SET and DET at Fertilitetscentrum, Gothenburg, Sweden, during the period 1998–2017

results with the USA shows a continuous increase in delivery rate to 35%; however, the MBR is still around 20% (3).

The significant health consequences of such high MBR are obvious, and the cost for the American society is very high [2, 7].

If Sweden had continued to transfer two embryos in the majority of patients, one could have expected an increase in the delivery rate similar to the USA. However, considering the unchanged delivery rate at an acceptable level and the dramatic decrease in the MBR with decreased risks for the IVF children, SET seems preferable, both from the patient's and the society's point of view.

Result from Sweden, with an overall SET frequency of almost 70% resulting in a multiple birth of less than 6% without reducing the overall birth rate, as well as published cohort studies, indicates how important it is to choose the correct embryo when implementing SET. This is because SET often is performed when a good quality embryo is available from a cohort of embryos. DET is, therefore, only used on patients who have a worse embryo quality, are older, and/or have many previous failed cycles. By using SET on patients that run a high risk of a multiple pregnancy, you, therefore, reduce this risk significantly. Methods for embryo selection have improved during the last decade or so using mainly morphological markers to increase the power of the embryo selection. Although improved embryo selection has been pivotal in maintaining a high pregnancy rate despite introducing SET, improved embryo culture media, blastocyst culture, and general improvement within the IVF clinics have also played a big part.

A big factor that is affecting the decision to use SET or DET is if a clinic has a well-established and successful cryopreservation program. With the introduction of vitrification in particular and overall improvements in general, the importance of the embryos that are cryopreserved has increased dramatically over the years. According to the Swedish registry, the proportion of frozen embryo transfers (FET) increased from being only 50% of all fresh cycles in 2005 to almost equal in 2014. The results have also increased dramatically almost surpassing the fresh results in 2015. Implementing a successful SET program is therefore very much reliant on being able to successfully cryopreserve viable surplus embryos and an important argument both for the couple and regulatory bodies.

60.5 Conclusion

Successful implementation of SET requires that everybody involved in the IVF treatment perform at their best, and some authors claim that the degree of SET performed at a particular center is the best measure for performance of that center [28]. Although there are pros and cons with SET, it is likely that its use will increase in IVF as major benefits can be achieved both for the child and mother but also for the society as a whole.

Review Questions

1. Why should SET be performed?
2. Who is the ideal SET patient?
3. Are there clinical situations when DET should be performed?

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Embryo Transfer: Techniques and Troubleshooting

Jutimala Bhattacharyya, Sankalp Singh, Madhab C. Das, and Kanna Jayaprakasan

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Learning Objectives

1. List the variables which could affect the success of the embryo transfer.
2. What are the different methods of embryo loading?
3. What is the role of mock transfer in IVF-ET?
4. Which is the optimal site for placement of the embryos and why?
5. What is a difficult embryo transfer and how would you overcome it?

61.1 Introduction

The procedure of embryo transfer (ET) is the crucial last step of a long arduous journey of the IVF process. What seems to be easy and simple is the most crucial event in the sequence of events that encompass an assisted reproductive technology (ART) cycle. The goal of a successful embryo transfer is to place the embryos in an atraumatic manner to an area of maximum implantation potential (MIP) to maximize the chance of a pregnancy. The idea is to avoid damage to the embryos as well as the endometrium and to avoid too much of manipulation that can stimulate uterine contractions and result in expulsion of the embryos from the uterine cavity. These may be the potential reasons of a failed transfer in spite of transferring top-quality embryos into a receptive endometrium. Hence, over the last decade, a lot of interest has been diverted to the embryo transfer procedure as an important determinant of IVF success. This chapter is an endeavour to review the technique and optimize the various aspects of embryo transfer to help maximize the IVF success.

61.2 Technique of Embryo Transfer

In general, the procedure begins with the patient being advised to fill their bladder to a comfortable status. A comfortably full bladder is one which is full enough to allow excellent visualization of the endometrium on ultrasound (US) and the straightening of the uterocervical (UC) angle without causing any discomfort to the patient. Its role is enhanced in straightening the uterocervical angle specially in an acutely anteverted uterus. This potentially minimizes the chances of cervical trauma and uterine contractions which are common if a tenaculum is used to grasp the cervix in order to straighten the uterocervical angle [1, 2].

Analgesia or anaesthesia is not necessary in most cases of transcervical ET. The procedure is performed under sterile conditions; the patient is placed in a dorsal lithotomy position with legs supported on a stirrup and draped with sterile towels. The cervix is visualized by placing a Cusco's speculum and cleaned with cotton swab. Some clinicians soak the cotton swab in saline solution or culture medium. Cleaning the cervix and vagina aims to clear the excess mucus of the cervix and possibly reduces bacterial contamination to some extent. Vaginal antiseptics are not recommended at ET due to the possibility of potential embryotoxicity. It is appropriate to remove excess cervical mucus as embryos may stick inadvertently to the mucus and get dragged out during removal of the inner catheter following transfer. Cervical mucus can be removed with a sterile cotton ball soaked in normal saline or culture medium. Additionally, cervical mucus can be aspirated using a sterile tuberculin syringe.

There are various methods of embryo transfer techniques, which are usually done under ultrasound guidance. Some clinicians use the direct embryo transfer technique (preload), where the catheter, already loaded with embryo/s, is passed through the cervix into the uterus. In the afterload technique, an empty catheter is passed to the level of the lower uterine segment under ultrasound guidance to a point where the inner catheter enters the endometrial cavity, typically about 5 cm. The inner sheath is slowly removed, leaving the outer sheath just at the internal os. A second inner sheath is loaded by the embryologist, who then assists the transfer in threading the inner sheath into the catheter. The clinician then advances the inner catheter into the uterus to deposit the embryo/s.

The tip of the ET catheter is traditionally placed 1.5–2 cm below the fundus and the embryos ejected as slowly as possible under US guidance, and the inner catheter is rotated and drawn out as gently as possible maintaining pressure on the plunger. Rapid withdrawal may create a negative pressure and result in the expulsion of embryos following the catheter. The inner catheter is passed back to the embryologist to check for retained embryos. However, pregnancy rates (PRs) were not compromised when the retained embryos were discovered and immediately retransferred into the uterine cavity [3].

Gentle manipulation during embryo transfer should be the rule as stimulation of the cervix causes the release of oxytocin, thus increasing uterine contractions. Holding the cervix with a vulsellum should be avoided unless as a last resort. Even while introducing the vaginal speculum, one should avoid pushing the cervix unnecessarily. Lesny et al. (1998) demonstrated that touching the uterine fundus can initiate waves of uterine contractions from the fundus to the cervix that may expel out embryos. As a general rule, embryo transfer should be a simple and painless procedure. The mere presence of the transfer catheter might be one of the factors that can trigger uterine contractions [4]. Some clinicians wait for a while before transferring the embryos, and some wait before withdrawal giving time to the uterus to stabilize. Martinez et al. in a prospective randomized study found no differences in pregnancy rates between removal of the catheter immediately after embryo deposit and after a 30 s wait [5].

Although there is no consensus on the optimal embryo transfer technique, there is evidence that certain methods in ET are associated with improved outcome after IVF. An abundance of literature suggests that the overall "ease" of the ET procedure is strongly correlated with the pregnancy outcome [1]. Although the degree of difficulty or ease of transfer is subjective, a study conducted by Candido et al. suggested that the degree of difficulty in embryo transfer after IVF/ICSI was an independent factor for predicting pregnancy and that an easy or intermediate transfer resulted in a 1.7-fold better pregnancy rates than a difficult one [6].

61.2.1 Degree of Difficulty of Transfer

- Easy – Smooth, without any instrumentation, the catheter was clean of blood.
- Intermediate – Primary catheter met with some resistance, leading to the use of a tenaculum and/or use of outer sheath following which embryo transfer was smooth without any blood contamination.
- Difficult – Any one of the conditions where greater resistance was met, the procedure was time consuming, caused discomfort to the patient, there was a need to change to a harder catheter, involve additional instrumentation such as uterine sounding or cervical dilatation or there was blood in any part of the catheter.

One mechanism by which a difficult or traumatic transfer can hinder implantation is by inducing uterine contractions caused by fundal contact or cervical manipulation which releases oxytocin and prostaglandins. The presence of blood on the catheter is also associated with decreased pregnancy rates and a higher incidence of retained embryos [7]. In a recent study, Mohammad E. Ghanem et al. (2016) concluded that cervical traction and blood on the outer sheath do not compromise clinical pregnancy rate or implantation rate [8].

61.3 Preparing the Patient

Appropriate assessment of the uterine cavity by ultrasonography (USG) prior to IVF cycle helps in the detection of polyps and fibroids causing cavity distortion. Many studies have favoured the hysteroscopic removal of polyps due to higher pregnancy rates that follow the procedure. A polypectomy done closer to ET, the interval being as short as 2–16 days, showed high pregnancy rates possibly due to endometrial damage induced by the procedure followed by a massive inflammatory response and the secretion of cytokines that play a vital role in implantation [9].

61.3.1 Ultrasound Measurement of the Uterocervical Angle Before Embryo Transfer

The uterocervical angle gives us an idea about the direction and length of the uterine cavity and is measured by ultrasonography. It is measured from the external os to the fundus of the uterus. A midplane longitudinal section is obtained, and the angle between a line joining the external cervical os and the internal cervical os and a line joining the internal cervical os and the uterine fundus is the uterocervical angle.

HN Sallam et al. in a prospective controlled study measured the uterocervical angle by a transabdominal USG and concluded that moulding the transfer catheter according to the UC angle significantly increased implantation and clinical pregnancy rates compared to the "clinical feel method" and reduced the incidence of difficult and bloody transfers. Patients with larger angles greater than 60° had significantly lower pregnancy rates compared with those with no angle. It was necessary to measure the angle immediately before the transfer as it was found during preliminary studies that the uterocervical angle changes with the state of the bladder. It is suggested that the patients with a uterocervical angle more than 60° should have a very full bladder for easy negotiation of the ET catheter [10].

A mock transfer or trial transfer prior to ET prepares the clinician for any difficulty in entering the uterine cavity during the actual embryo transfer especially in situations such as large degree of anteflexion/anteversion or retroflexion/retroversion, pinpoint external os, fibroids, previous surgeries causing stenosis of the cervical canal and uterine cavity distortions.

A trial or mock transfer can be done at any point before the actual transfer, but the two most common times are before ovarian stimulation and immediately before the actual ET. A catheter is advanced to the uterine fundus to measure the full length and direction of the uterine cavity and cervical canal, and notes are made regarding the direction and curve of the catheter and type of catheter used. The mock ET can be done blindly or under USG guidance if difficulty is encountered. If the trial reveals a too stenotic cervix to permit passage of a catheter, a cervical dilatation can be planned well in advance before ET in the form of mechanical dilatation, use of osmotic dilators or operative hysteroscopic shaving. Dilatation at the start of stimulation allows sufficient time for the endometrium to recover from any trauma, inflammation or bacterial contamination before ET. Lower pregnancy rates are associated with cervical dilatation done within 5 days of transfer. Dilatation done several weeks before ET appears to improve PRs. While mock ET may possibly provide useful information to plan future ET procedure, a mock transfer remote from the actual ET is done under different circumstances and may not be reflective of actual conditions encountered on the day of ET.

The "afterload technique" is a trial transfer done at the time of ET in which an empty Wallace catheter is passed to the level of the lower uterine segment under ultrasound guidance to a point where the inner catheter enters the endometrial cavity, typically about 5 cm. The inner sheath is slowly removed, leaving the outer sheath just beyond the internal os. Then a second inner sheath is loaded by the embryologist and handed over to the clinician who threads the inner sheath into the outer sheath and completes the transfer.

Indeed, there are multiple benefits to the afterload technique. Since the uterus often changes position during stimulation, a previous mock transfer may not be relevant on the day of ET. It allows the transferring physician to take the time needed to slowly and gently pass a catheter through the endocervix while the embryos remain in the incubator, thus facilitating an atraumatic transfer while limiting the time the embryos are exposed to room environment. In addition, the embryos will not pass through the initial inner sheath that is placed through the cervix. In theory, this would decrease

mucus contamination of the catheter, which has been proposed to adversely affect implantation either by contamination of the cavity or by causing retention or displacement of the embryos. The clinical pregnancy rate in the group with ET using the afterload technique was higher than in the direct ET group (52.4% vs. 34.9%) [11].

61.4 Preparing/Loading the Catheter

A lot of attention has been given to the embryo loading technique in the recent years in order to improve the ART outcome. Embryo loading is a stage of embryo transfer that is performed by a clinical embryologist and is one of the key steps which influences the success of the ART procedure. Although it seems simple and easy, it is one of the most delicate steps in embryo transfer. The embryo is drawn from the culture medium into the catheter, and the loaded catheter is then handed over to the clinician for ET.

In general, there are two distinct catheter loading techniques: the air-fluid method and the fluid-only method. The most commonly reported method has been found to be medium-air-embryo-air-medium [12].

A soft catheter is used, and the ET catheter is flushed with Hams F10 using a 1 ml syringe prior to the loading. Embryos are loaded in approximately 30 μl of medium that is flanked by small air bubbles. Air bubbles can help with ultrasound visualization of the ET catheter and proper placement of the embryo(s) [2]. The total volume of medium injected during transfer is 30 μl with the embryos in the most distal 10 μl.

The embryo catheter loading technique involves several clinically relevant variables: the choice of syringe, the type of catheter, the type and volume of transfer medium, the presence of air bubble, catheter loading speed and embryo placement in the catheter. However, the volume of medium that is transferred and the presence of an air bubble are particularly important and also the most controversial. Christianson et al. performed a worldwide web-based survey of 265 IVF centres in 71 countries [12]. The most commonly reported methods of embryo loading were medium-air-embryo-air-medium (42%) followed by medium in the catheter with embryo at end (20%) and medium-air-embryo (15%). In 68% of centres, the final volume of the catheter was up to 0.3 ml with only 19% using 0.3–0.5 ml and 1% using 0.5–0.7 ml. Almost all centres (97%) prefer a catheter with the orifice on its top. The majority of the centres (82%) wash the catheter prior to loading the embryos.

Some clinicians are of the opinion that the presence of an air bubble in the ET catheter can be useful for identifying the embryos and the medium during ultrasound-guided ET (UG-ET) [13]. In addition, the use of air bubbles to flank the embryos from both sides can protect the embryos from the cervical mucus and accidental discharge before entering the endometrial cavity [14]. However, another school of thought believes that even a small amount of air in the uterus is a nonphysiological factor that has a deleterious effect on the embryos and implantation [15]. It was found that the presence of air increased the likelihood of the embryo moving up towards the syringe, thus increasing the risk of embryos being retained within the catheter.

A systematic review and meta-analysis conducted by Abou-Setta AM has found no difference in pregnancy rates with or without air bubble in the ET catheter during transfer [16].

Halvaei et al. compared embryo catheter loading techniques involving loading directly from the culture microdrop versus loading from the transfer dish and did not find any significant difference in terms of pregnancy rates between these two methods [17]. In group A, the embryos were drawn directly into the ET catheter from the culture microdrop under the oil. In group B, the embryos were transferred from the culture microdrop into G2 medium in a centre well dish and then drawn into the ET catheter in order to wash off the oil. The rationale for drawing the embryos directly from the culture drop is growth factors that are released from the embryos during culture. The periimplantation embryos may produce some beneficial factors for stimulating development of both self and adjacent embryos. Factors like PAF, IGF1 and IGF2, LIF and EGF can be useful for embryo survival and development as well as implantation success. But the disadvantage of this method is contamination of the catheter tip with oil that layers the culture drop, which could be detrimental to embryo implantation. Although the catheter tip is washed in the transfer medium prior to ET, some amount of microdroplets may still remain on the catheter tip.

61.5 Placement of Embryo

Proper placement of the catheter tip and ejection of embryos is another important variable affecting the ET outcome but lacks a uniform universal guideline regarding the ideal location of placement of embryos. Traditionally, the tip of the catheter has been placed 5–10 mm from the uterine fundus. However, several recent studies have suggested that transfer further away from the fundus was more ideal. A transfer depth of 15–20 mm from the fundus may optimize implantation by avoiding the lower cavity where implantation is compromised and, at the same time, avoiding problems like endometrial trauma, uterine contractions and ectopic pregnancy associated with upper cavity transfers. Coroleu et al. in a prospective randomized controlled study found that depositing the embryos >15 mm from the fundus had a higher PR (60% vs. 39.3%) compared to within 10 mm distance from the fundus [18]. Embryos deposited less than 5 mm from the fundus have a lower pregnancy rate and higher ectopic pregnancy rate.

Pope et al. determined the transfer distance from the fundus (TDF) and found that the pregnancy rates were higher when the catheter was anywhere from 5 mm to 27 mm from the fundus instead of being right at the fundus [19]. TDF was calculated by subtracting the depth of catheter insertion (DCI) from the cavity depth (CD) measured by ultrasound.

Lambers et al. assessed the position of the air bubble flashes at the time of ET and found that the air bubbles in the fundal half of the endometrial cavity were associated with higher pregnancy rates compared to the ones in the lower half of the cavity (43% vs. 24.4%) [20].

In a recent study, the location of air bubble flashes at the end of 60 min after ET was evaluated, and it was found that the implantation and pregnancy rates among patients with air bubble flashes located <15 mm from the fundus were significantly higher than those with embryo flashes located >15 mm from the fundus [21].

Some investigators have suggested that the desired location should take the length of the uterine cavity into account rather than a fixed reference point such as transfer distance from the fundus. A randomized controlled trial (RCT) conducted by Franco JG in 2004 showed similar implantation rates and clinical PRs in the upper and lower uterus but significantly higher rates in the mid-cavity. Mario Cavagna et al. studied the implantation sites after embryo transfer into a standard central area of the uterine cavity, and they found that the chances of implantation were maximum in the mid-cavity. Most data in literature support ET in the midportion to the lower midportion of the uterus, avoiding high transfers in the uterus [22, 23].

Another concern with ET is the possibility of expelled embryos. Mansour et al. using methylene blue demonstrated the dye was visualized at the external os of the cervix in 42% of cases. Embryos can move back into the cervix owing to capillary action whereby fluid injected actually trailed the catheter as it was withdrawn [24].

Mansour et al. described another study in which the blades of the speculum were collapsed on the lower uterine segment after ET while the catheter was still left in place, to minimize the fluid to be expelled out through the cervix.

Madani et al. (2010) studied the effect of pushing 0.2 ml of air into the catheter immediately following ET to prevent embryo expulsion. By pushing air gently, they have tried to generate a positive air pressure in order to stop embryos from backtracking with the catheter removal on the creation of a force of waves by uterine contractions and thus reduce the rate of embryo expulsion [25].

Use of Embryo Glue which is a useful and available embryo transfer medium with high concentration of hyaluronic acid was reported to be associated with higher implantation rates in recurrent implantation failures [26]. Hyaluronic acid also known as implantation-enhancing molecule [27] improves the chances of implantation by generating a viscous solution that increases cell-to-cell adhesion and cell-to-matrix adhesion and thereby prevents embryo expulsion. In a systematic Cochrane review [27], hyaluronic acid was found to increase the chances of pregnancy but also increased the chances of multiple pregnancies, which is a less positive result. RCTs specifically looking at its use during single embryo transfer and at cleavage stage and blastocyst embryos are warranted to draw any definitive conclusion (Table 61.1).

Table 61.1 Key elements for a successful ET

Goal	Protocol
Easy, atraumatic transfer without blood or mucus	Trial transfer, US, soft catheter
Proper placement guidance	Inject embryos slowly 1.5 cm from the fundus, US
Minimize embryo stress	Minimize transfer time, control temp./PH
Negotiate a difficult/stenotic Cx	Precycle laminaria, malleable stylet, ultrasound
Optimize implantation, minimize contractions	Day 5, FET, avoid trauma to Cx or fundus

Schoolcraft [2]

61.6 Variables Influencing the Success of Embryo Transfer

The success of ET depends not only on embryo quality and endometrial receptivity but also on the technique of embryo transfer. Several variables play a role in the success of a transfer. Literature strongly suggests that an easy atraumatic transfer, type of catheter used and use of ultrasound guidance can significantly improve implantation and pregnancy rates. Limited evidence also supports trial transfers, experience of the physician, removal of cervical mucus and deposition of embryos in the mid-cavity in the minimum possible amount of time.

61.6.1 Uterine Contraction in Embryo Transfer

Immediate or delayed expulsion of embryos after transferring them into the uterine cavity has always been of concern in assisted reproduction, and one of the most important causes of this is uterine contraction during embryo transfer, especially difficult embryo transfers. Uterine contractions can be present before, during or after embryo transfer and are usually triggered by instrumentation of the cervix, trauma to the endocervix and endometrium or the tip of the transfer catheter touching the uterine fundus. Even alteration in the normal contraction pattern may cause expulsion of the embryos from the uterine cavity [28]. Fanchini et al. investigated the possible consequences of uterine contractions just before ET as visualized by ultrasound on IVF-ET outcome. Approximately, 30% of patients who undergo ET experience uterine contractions. The higher the frequency of uterine contractions, the lower the chance of implantation and clinical and ongoing pregnancy rates. Patients with uterine peristalsis <3 waves/min had a higher chance of pregnancy compared with those more than 3 waves/min (uterine contractions >5/min have been significantly associated with worst IVF outcomes) [29, 30].

Holding the cervix with a tenaculum was found to stimulate uterine junctional zone contractions affecting implantation of the transferred embryos, the mechanism being the release of oxytocin and prostaglandins. When the tenaculum was used, it was temporarily associated with an elevation in oxytocin levels which remained elevated throughout the embryo transfer procedure, and in the absence of tenaculum, no increase in oxytocin levels was observed [31, 32]. Therefore, holding the cervix by a vulsellum or any other instrumentation should be completely avoided except in difficult cases. Even while introducing the vaginal speculum, care should be taken to avoid unnecessary pushing of the cervix [33].

The use of soft catheters should be the first choice in order to avoid injury of the endocervix or endometrium. When using the outer sheath, it is important to keep it just proximal to the internal os. Touching the fundus starts strong random waves of contraction in the fundal area which can expel embryos. Sometimes, the mere presence of the ET catheter might be one of the factors that can trigger uterine contractions [34]. Ultrasound-guided ET has the advantage of visualizing the catheter placement in the endometrial cavity minimizing the chance of touching the fundus. A comfortably full bladder in USG-guided ET helps in the correction of acute anteflexion of the uterus, and the use of a tenaculum can be avoided. Some authors have suggested that it is preferable to wait for the release of embryos till the contractions pass off or to wait before the withdrawal of the catheter after transfer so that the uterus can stabilize [35].

The role of adjuvants that prevent uterine contractions has been considered to improve ET outcome. Tocolytics, prostaglandin synthetase inhibitors and vaginal progesterone as uterine relaxants have been suggested. Other than vaginal progesterone, no other drug has been proven to be useful in preventing uterine contractions. Progesterone administration on the day of oocyte retrieval induces a decrease in uterine contraction frequency on the day of ET. During the luteal phase of ovarian stimulation, uterine contractility decreases progressively and reaches a near quiescent status 7 days after hCG administration, that is, at the time of blastocyst transfer [36]. It is possible that such a uterine relaxation assists blastocyst implantation. Moraloglu et al. concluded that oxytocin antagonist atosiban given intravenously 30 mins prior to ET increased the implantation rate and pregnancy rate and decreased the miscarriage rate. The rationale behind it is that higher concentration of circulating oestradiol due to controlled ovarian hyperstimulation (OHS) may be a causative factor for increased uterine contractions by promoting the oxytocin effect in non-pregnant uterus through the oxytocin receptor gene expression in the myometrium [37]. In a recent study by Kim et al., it was observed that the oxytocin antagonist on the day of ET improves the implantation rate but not the clinical pregnancy rate or miscarriage rate [38]. However, in a study by Lan UT et al., atosiban has been shown to improve implantation and pregnancy rates in patients with recurrent implantation failure [39]. As a general rule, embryo transfer should be a gentle and painless procedure, avoiding any manipulation that may trigger uterine contractions so as to improve the ET outcome.

61.6.2 USG-Guided ET

The clinical touch technique of embryo transfer has been gradually replaced by USG-guided embryo transfer since it is a blind procedure and the chances of catheter indenting or becoming embedded in the endometrium reduce the chance of implantation. Clinicians must rely on tactile assessment, and inadvertent contact of the catheter with the fundus may lead to uterine contractions and expulsion of embryos. Since first described by Strickler et al. in 1985, numerous studies have been published evaluating the effect of USG-guided ET on pregnancy rates. A 2010 Cochrane review of 17 RCTs comparing ultrasound versus clinical touch concluded that ultrasound did increase the ongoing clinical PRs over clinical touch [40].

USG-guided ET is routinely used by most clinicians as it gives us an opportunity to visualize the ET catheter, the exact location of placement of embryos and the process of ejection of the embryos into the uterine cavity in the form of air bubble flashes. Ultrasound guidance is especially helpful in cases of the uterus distorted by fibroids or previous caesarean section scar defects or a severely anteflexed uterus. Direct visualization of the catheter at the cervico-uterine angle allows the physician to place an appropriate curve on the catheter and negotiate in a severely anteflexed uterus. A full bladder required for abdominal ultrasound also helps to straighten the cervico-uterine angle and facilitate entry of the catheter. Touching the fundus can easily be avoided with ultrasound, and one can be certain that the catheter is beyond the internal os in cases of an elongated cervical canal. Patients also seem to take great comfort and satisfaction in visualizing this final step of an often long and tedious process in the form of air bubble flashes. USG guidance has been associated with fewer ectopic pregnancies in some studies [41]. The role of USG has been extended to the use of 3D/4D or vaginal ultrasound during embryo transfer with promising initial results.

Some authors have suggested the use of ultrasound guided-ET (UG-ET) using transvaginal (TV) ultrasound over transabdominal (TA) UG-ET approach with encouraging results. TV UG-ET was originally reported by Hurley et al. followed by few more studies suggesting the advantage of TV UG-ET over TA UG-ET [42]. TA UG-ET typically requires a full bladder carrying potential for cramping, which could impact on outcomes. In addition, TA UG-ET also necessitates assistance to help with the ultrasound or placement of the embryo catheter. In contrast, TV UG-ET does not require the assistance of a sonographer, avoids discomfort of a full bladder, gives greater resolution of the uterocervical angle and can more clearly delineate the catheter tip than TA UG-ET but needs the use of a Kitazato long ET catheter [42].

However, TA UG-ET is the most widely used approach as the procedure is less complex compared to TV UG-ET with associated practical difficulty in doing a speculum and TV scan together. A recent RCT compared TA and TV UG-ET demonstrating no differences in overall pregnancy, clinical pregnancy, live birth and implantation rates between both groups [43]. Another study conducted by Bodri et al. yielded similar success rates when they compared TV UG-ET to TA UG-ET but with the advantage of increased patient comfort due to the absence of bladder distension [44].

The severely anteflexed uterus seemed better served with a TA UG-ET as the full bladder tended to straighten the cervico-uterine angle. Conversely, the retroflexed uterus appeared to be better served with a TV UG-ET. Lastly, a pre-transfer ultrasound on the day of the ET (much like a mock ET) served as a guide to decide the optimal ultrasound approach, particularly with hyperstimulated ovaries that may alter the cervico-uterine angle following oocyte retrieval.

Gergely et al. in 2005 proposed a novel method that used 3D/4D USG in the precise placement of embryos at the *maximum implantation potential (MIP) point*. The shape of the uterus is like an inverted triangle with the cervix and vagina at the apex and the fallopian tube openings at either end of the base. The MIP point is the intersection of two straight lines drawn downwards to the midline from the openings of the fallopian tubes. This point is important because in this part the uterine endometrium has the richest blood supply and this is the area where the implantation of naturally fertilized ovum occurs. Embryo transfer at the MIP point increased the implantation and pregnancy rates and reduced the requirement of transferring multiple embryos, thus reducing the chance of multiple pregnancy and chance of ectopic pregnancy. The MIP point varied from patient to patient depending on the size and shape of the uterus [45].

Lanlan et al. (2009) compared 2D ultrasonography with 3D sonography in embryo transfer and concluded that 3D sonography had a definite advantage over 2D in terms of uterine cavity depth measurement and catheter placement, thus improving implantation rates and clinical pregnancy rates. 3D ultrasound has enabled accurate, non-invasive diagnosis of uterine anomalies and more accurate estimation of the catheter tip position in the uterine cavity specially in those with abnormal uterine cavity (arcuate, bicornuate and sub-septate uterus) [46].

61.6.3 Choice of Catheters

Soft catheters are preferred over rigid ones as they are less likely to traumatize the cervix and endometrium and to invoke any uterine contractions. The commonly used soft catheters (e.g. Wallace) have a softer inner cannula and a stiffer outer sheath. In two meta-analyses of RCTs, the use of soft catheters was associated with a higher PR than firm catheters [47, 48]. Alternatively, the outer sheath from this trial catheter can be left in place, either routinely or with difficult cases, while the soft inner catheter is loaded with the embryos and advanced through the sheath to the correct placement. This method, sometimes referred to as the afterload technique, may also decrease mucus contamination of the catheter. Occasionally, resistance to pass the soft inner cannula into the uterus usually at the level of internal os is encountered, and in these cases the stiffer outer sheath is advanced in the cervical canal to negotiate the resistance with the inner cannula which can now be advanced into the uterine cavity. A malleable stylet device can be used with some soft catheters to negotiate a difficult internal os. It was demonstrated that the use of this device compared with a soft catheter alone did not decrease clinical pregnancy or implantation rates. The inner stylet is then removed, and the softer cannula with embryos loaded in it is fed through the outer sheath to advance into the uterine cavity for transfer. When using the malleable stylet, it is important to leave the outer sheath just proximal to the internal os. Keeping it in this location before inserting the inner soft catheter was found to significantly improve clinical pregnancy rates. In some cases, insertion of the catheter is difficult owing to cervical stenosis. Different approaches have been undertaken to alleviate this issue, including cervical dilation at the time of retrieval. However, this was found to lower subsequent pregnancy rates. Dilation has also been accomplished several weeks before ET and was found to improve outcomes.

61.6.4 Time from Loading to Embryo Transfer

The time interval from loading the ET catheter to depositing the embryos in the uterus should be minimum as possible as embryos are vulnerable to exposure to environmental temperature, light or other agents in the catheter. A longer time interval of more than 120 s carries a poor prognosis [49]. This may be related to how long the embryos are "outside the incubator" as well for the difficulty of ET. However, a study conducted by Ciray HN et al. showed no adverse effect of the duration of the procedure where good-quality embryos were transferred and duration of the procedure lasted up to 7.5 min [50].

61.6.5 ET Speed

A study of fluid dynamics during embryo transfer conducted by Cezary Grygoruk et al. found that an increase in injection speed during embryo transfer increased the shear stress, dynamic stress and velocity differences acting on the embryo. The narrowing of the ET catheter by 20% amplified the transferred fluid velocity by 78%. An embryo positioned in proximity to the catheter wall was exposed to considerably higher shear stress, dynamic pressure and velocity difference than an embryo in the centre of the catheter lumen. Hence, transfer of an embryo should be conducted gently and with minimum injection speed. Any narrowing of the catheter lumen should

be eliminated, and the embryo should be kept far from the catheter wall during injection of the transferred load [51].

There is some evidence that local pressure fluctuations during ET may cause cell damage and influence embryo viability. The percentage of embryos that were shrunken and/or collapsed and undergone apoptosis was higher in the rapid injection group. That is reason enough to propose that ET should be performed as slowly as possible with minimum ejection speed to avoid exposing the embryo to steep pressure gradient. Additionally, rapid injection may possibly promote ectopic pregnancies [52].

61.6.6 Volume and Type of Media

Commercially available media are made up of various concentrations of ions, amino acids and carbohydrates, but still there is lack of evidence of a superior transfer medium over the other. Protein concentration and viscosity have not been demonstrated to impact pregnancy outcomes. Addition of fibrin sealants and a glycosaminoglycan, hyaluronic acid, to the transfer medium has been evaluated in order to see whether it improves the IVF outcome. A fibrin sealant added to the medium was studied in two RCTs. One of them showed no significant improvement in PR, and the other showed a benefit in older patients [53]. A recent Cochrane study reviewed the role of adherence compounds in embryo transfer media and found no evidence that fibrin sealants increase pregnancy rates. For hyaluronic acid, evidence of a positive treatment effect was identified in six trials that reported higher live birth rates [27].

Another matter of controversy is the volume of medium of the ET catheter. A very small volume of medium (<10 μl) along with air bubbles had a negative effect on the implantation and pregnancy rate [54]. Therefore, a volume of 10–30 μl has been proposed since high fluid volume may predispose to ectopic pregnancy. Also, a large volume of transfer medium >60 μl can increase the chance of dislocation of transferred embryos from the uterus into the cervix. On the other hand, Montag et al. compared 40–50 μl and 15–20 μl and found that high fluid volume increased both implantation and pregnancy rates [55]. However, a recent study by Marjan Omidi et al. did not show any significant differences in the pregnancy and implantation rates between 35–40 μl and 15–20 μl transfer volume [13].

61.6.7 Contamination of ET Catheter Tip with Blood or Mucus

Cleaning the cervix with normal saline and removing cervical mucus are necessary or else it may plug the ET catheter causing difficulty in delivering the embryos especially with such a small volume of culture media to inject with the embryos. It may so happen that cervical mucus introduced into the uterine cavity may surround embryos and dislodge them from their location dragging them out during the withdrawal of the catheter. If mucus is pushed higher into the uterine cavity while introducing the catheter, it may interfere with implantation. Studies have shown that embryos were more likely to be retained when the embryo transfer catheter was contaminated with mucus or blood. In a preliminary study, blood or mucus on the tip was associated with a significantly lower pregnancy outcome [56]. Contamination of the catheter with blood may be a marker for difficult ET and has also been linked to poor ET outcomes. Goudas et al. retrospectively demonstrated a clinical pregnancy rate of 50% with no blood, and this rate fell by half when a small amount of blood was present at the catheter tip. Pregnancy rates fell down to 10% when there was significant amount of blood [57].

61.6.8 Role of Rest After ET

Embryo transfer is the crucial last step in assisted reproductive treatment. To achieve optimum outcome, both the clinician and the patient will do everything conceivable to improve the chances of success. This includes use of various hormonal preparations, many nonhormonal medicines, etc. The patients also try to follow medical direction to optimize the chances of success, including use of some non-medical advice. Bed rest after embryo transfer is one such issue, which has traditionally been viewed by patients as an important part of their effort to increase the chances of implantation. This is contrary to the reasonable body of evidence showing no role of prolonged bed rest after ET as far as implantation rate is concerned.

During initial days of ART, it was believed that prolonged bed rest after embryo transfer will help avoid expulsion of the embryo. There has been a plethora of advice regarding the kind of bed rest ranging from prone position for 4 h to complete bed rest for 24 h. There has been an occasional clinic advising bed rest until the pregnancy test. A postal survey of 80 practitioners from 40 units regarding attitudes towards the factors affecting embryo transfer practice in the UK by Balen et al. showed that prolonged bed rest after ET is the least important factor to influence the outcome [58].

The available evidence shows that bed rest has minimal role in the success of IVF treatment. The study by Giuseppe Botta and Gedis Grudzinskas (1997) comparing 24-h bed rest versus 20-min rest has not shown any statistically significant difference in IVF-ET outcome. A total of 182 patients were studied and matched for all the other variables affecting the outcome. There has not been any difference in pregnancy rate, miscarriage rate and multiple pregnancy rate in both groups [59].

Another single-centre prospective study on 406 patients by Bar-Hava et al. (2005) comparing immediate ambulation with bed rest for 1 h shows no difference in pregnancy rate [60]. Woolcott R et al. investigated in a small prospective study in 1998 on 93 patients undergoing 101 consecutive

embryo transfers to assess whether standing upright immediately after the procedure affected the movement of embryo-associated air bubble as an indirect way of judging potential movement of embryo.

Transvaginal ultrasound-guided embryo transfer was done with a repeat USG in standing position immediately post-transfer. There was no movement of air bubble in 94.1% (95/101), in 4.0% (4/101) only <1 cm movement and 1–5 cm movement in 2.0% (2/101) of transfers. No movement of air bubble is noted out of the uterine cavity into the cervix or fallopian tube. The authors concluded that standing shortly after embryo transfer does not have a significant role to play in final embryo position [61]. In fact, a recent study by Sharayu Gaikwad et al. (2013) on 240 patients undergoing first-time embryo transfer with ovum donation in a prospective, randomized, parallel assignment, controlled trial has shown statistically significant higher live-born infant in the no rest group than the group resting for 10 min [62].

A systematic review and meta-analysis of four RCTs including 757 women showed no improvement in pregnancy and live birth rates but reduced implantation rate with bed rest. The findings are concordant with previously published literature suggesting that bed rest is not beneficial following ET. Rather, it might negatively affect the outcome of IVF/ICSI cycles via probable stress/anxiety mechanisms [63]. Mert Küçük (2013) also concluded that there is insufficient evidence to support bed rest after ET; instead, accumulated data indicate restriction of physical activity may actually be detrimental and associated with worse outcome [64].

61.6.9 Role of Antibiotics After ET

Use of antibiotics has been proposed by some to improve the chances of success following embryo transfer, as it is believed that the presence of infection at the time of ET would reduce the possibility of implantation. However, a recent randomized controlled trial of prophylactic antibiotics before embryo transfer by Brook N et al. [65] on 350 patients showed no such association as far as clinical outcome is concerned. There was, however, a significantly lower chance of growth of bacteria in the ET catheter specimen after the use of antibiotics (49.4 vs. 62.3%, RR = 0.79, 95% CI = 0.64–0.97, $p = 0.03$). The study showed no difference in clinical pregnancy rates between the two groups (36.0 vs. 35.5%, $p = 0.83$) though it revealed a significant ($p = 0.03$) association between the level of bacterial contamination and clinical pregnancy rates. Clinical pregnancy rate was 47.2% when the catheter tip was sterile; in contrast, CPR gradually diminished from 40% to 15.8% with increasing level of growth of Gram-positive bacteria, and it was 18.2% when the catheter tip had grown Gram-negative bacteria. The likely explanation for this association is that bacterial contamination reduces the implantation potential of the embryo as well as its effect on the endometrium. The authors of this study did not recommend routine use of prophylactic antibiotics before ET.

61.7 Troubleshooting During Embryo Transfer

61.7.1 Difficult ET

Difficult embryo transfer is seen in 5–7% of patients having assisted reproduction treatment, and in about 1%, it might be extremely difficult to perform ET transcervically even by experienced practitioners. There is no universally accepted definition of difficult ET. A recent study defined very difficult embryo transfer as one which was being labelled so by an experienced embryo transfer practitioner (who had performed at least 100 embryo transfers per year for 2 years) and that took a longer time than usual and required two or more of any of the following manoeuvres: use of the tenaculum and/or change of the embryo transfer catheter and/or use of a stylet and/or reloading of the embryos or cancelling the procedure or cancellation of fresh embryo transfer and opting for frozen embryo transfer after cervical dilatation and hysteroscopy [66]. A recent study of 7714 ETs evaluating difficult ETs suggested objective classification of ET difficulty into four types: type 1, smooth or direct ET without additional manoeuvres; type 2, afterloading with an outer catheter sheath; type 3, afterloading with a Wallace stylet; and type 4, subsequent use of the tenaculum and/or poor ultrasound visualization [67].

To have a successful IVF procedure, doing an atraumatic ET is of utmost importance. The entry of the ET catheter inside the uterine cavity may prove difficult in the presence of associated factors such as cervical polyps, cervical fibroids, pinpoint external os and cervical deformation/stenosis either due to congenital anomalies or those acquired from a previous surgery. Easy or intermediate transfers have been shown to have a 1.7-fold higher pregnancy rate than difficult transfers (95% confidence interval 1.3–2.2) in a comparative study of 4807 ETs [68]. A meta-analysis performed in 2013 showed a lower clinical pregnancy rate for a non-easy transfer demonstrating a considerable harm (RR = 0.67; 95% CI = 0.51–0.87; $p = 0.003$). The authors concluded that there is a low-quality evidence to suggest that a difficult embryo transfer reduces the chance of achieving a clinical pregnancy. Unfortunately, none of the studies evaluated live birth rate [69].

It has been suggested trial transfer, or mock transfer, helps to evaluate how the ET is going to be and makes an appropriate procedure plan. It is ideally performed in the month before the actual IVF cycle. It helps one assess the ease/difficulty in the passage of a transfer catheter via the cervical canal into the uterus, the cervico-uterine length and the position of the uterus (anteverted or retroverted) and thus can guide the clinician during the process of actual ET. Any abnormalities found can then be treated accordingly. For example, a cervical stenosis can be successfully overcome by doing a cervical dilatation prior to starting ovarian stimulation [32]. However, there are studies questioning the benefit of a trial transfer claiming that it might not be reflective of an easy or difficult actual ET since the uterine position at actual ET might be different from that at the mock ET due to enlarged ovaries from ovarian stimulation [70]. There is no

overwhelming evidence suggesting that performing a trial transfer prevents a difficult embryo transfer. Conversely, Mansour et al. in their RCT of 335 women concluded that trial transfer conducted in the cycle preceding the actual IVF-ET cycle significantly improved the clinical pregnancy rate from 13.1% to 22.8%, thus avoiding unexpected difficult ET [71]. It is generally considered a good practice by most of the clinics to do a trial transfer prior to IVF-ET.

Conscious sedation has been used as an alternative method during difficult ET [72]. Further studies are required to confirm the efficacy of sedation in women with difficult ET.

61.7.2 Endometrial Cavity Fluid in IVF

In an IVF cycle, 2–3% of women have presence of fluid in the endometrial cavity on the day of oocyte retrieval. Excessive amount of fluid in the endometrial cavity (≥3.5 mm in anteroposterior diameter in the sagittal plane) on the day of oocyte retrieval was found to have a negative impact on the outcome of IVF-ET [73]. In 25% of cases, this fluid collection is found to be due to coexisting hydrosalpinx, while in others, it could be due to other tubal pathologies, subclinical endometrial infection or abnormal endometrial development.

A recent meta-analysis of six studies evaluating 5928 ART cycles showed that the pregnancy rate was significantly lower in the group with ECF than in the group without ECF (OR = 0.74, 95% CI = 0.55–0.98) [74]. In view of the suboptimal cycle outcome, deferring the fresh embryo transfer, elective cryopreservation of all the embryos followed by their replacement in a frozen thaw cycle is considered to be a better option when fluid is present in the endometrial cavity. An alternative option explored for women with excessive ECF is aspiration of the ECF before embryo transfer. Griffiths and colleagues reported a case series of five women in which they removed 5–6 mL of ECF with an embryo transfer catheter immediately before embryo transfer leading to pregnancies in all of them [75]. A much larger prospective matched control study of 132 women undergoing IVF found similar implantation rates in women who underwent ECF removal compared to controls [76]. Few other studies failed to reproduce these positive results. Hence, at the moment, ECF aspiration as a modality of treatment for patients with ECF should be reserved in couples who do not want to postpone embryo transfer.

61.7.3 Cervical Mucus at the External Os

Despite significant research, it is not yet clear whether cervical mucus should be removed or not prior to ET. On one hand, it is thought to help in easy insertion of the ET catheter providing a slippery coating as a natural lubricant over the cervical canal, while on the other hand, it seems to have certain detrimental effects.

Cervical mucus may clog the tip of the transfer catheter, thereby causing difficulty in pushing the embryos into the endometrial cavity. Mansour and colleagues did an interesting study by doing intrauterine instillation of methylene blue dye as a surrogate for culture media in a mock embryo transfer model [77]. In this study, the patients underwent mock ET before and after cervical mucus aspiration. Their results demonstrated that the dye extrusion rate at the external os was higher when the cervical mucus was not aspirated compared to when it was aspirated (57% and 23%, respectively).

The tip of the catheter might get contaminated by the microorganisms present in the cervical mucus. The positive culture rate of the cervical mucus and catheter tip was 70.9% and 49.1%, respectively, in a study by Egbase and colleagues. The clinical pregnancy rates for catheter tip-negative women were higher when compared to catheter tip-positive ones (57.1% and 29.6%, respectively) [78]. Careful cleaning of the cervix prior to embryo transfer is therefore considered as a norm rather than exception.

There is increased incidence of retained or expulsion of embryos because the embryos stick to the cervical mucus which dislodge them from their original placement. Nabi and colleagues found that the chance of embryo retention is nearly six times more likely when the embryo transfer catheter is contaminated with mucus (3.3 vs. 17.8%) [3].

While there are reported detrimental effects of excessive cervical mucus as above, there are concerns that the procedures of removing cervical mucus can possibly stimulate uterine contractions or cervical bleeding. Studies have evaluated two ways to tackle the cervical mucus – either an aspiration of cervical mucus using a syringe and culture media or to remove it manually using wither cytobrush or a cotton swab. In a prospective controlled trial of 286 women, Eskandar and colleagues evaluated the effect of cervical mucus aspiration prior to ET on the clinical pregnancy rate [79]. The mucus-aspirated group showed a significantly higher CPR (63/143) than the control group who did not have cervical mucus aspiration (38/143) (OR = 2.18, 95% CI = 1.32–3.58). This was seen despite the fact that there were a higher proportion of easy transfers in the latter (OR = 3.00, 95% CI = 1.05–8.55).

The effect of removal of cervical mucus by a cytobrush prior to embryo transfer was studied in a single-blind randomized controlled trial of 317 couples assessing live birth rate as the primary outcome. Live birth was not significantly different between the two groups (24% vs. 21% in study and control, respectively). They concluded that it was unlikely that pretransfer cervical mucus removal has a significant effect on live birth rate [80].

The biggest study till date was by Moini and colleagues who evaluated 530 women undergoing fresh embryo transfer following IVF. The intervention group had cervical mucus removed using a sterile cotton swab, while the control group had routine ET without removing the mucus. The intervention group had significantly higher clinical pregnancy rate, implantation rate and live birth rate compared to the control group. The logistic regression analysis gave an odds ratio of 2.3 (95% CI = 1.55–3.4) for pregnancy in the intervention group as compared with the control group [81]. As of today, cervical mucus removal should be a part of standard practice before embryo transfer.

61.7.4 Retained Embryos in the Catheter

Retained embryo following an initial transfer attempt is a fairly uncommon event, but certainly is the one capable of creating anxious moments for the clinicians, embryologists and patients. Most of the studies quote an incidence of <3%; however, there are few who quote up to 10%. The reported but yet not conclusively proven risk factors of retained embryos at ET include:

1. Higher number of embryos transferred
2. Cleavage-stage ET
3. Clinician's experience
4. Transfer technique
5. Difficult ET
6. Contamination of the ET catheter with mucus or blood

The timing of the appearance of the problem of retained embryo precludes performing an RCT. Hence, the available evidence largely comprises of retrospective analysis. Various such studies have shown that the pregnancy rate is not compromised if retained embryos are immediately recognized and retransferred into the uterine cavity [3]. It is unlikely that any evidence to the contrary is possible in the near future.

61.7.5 Fresh ET vs. Frozen ET

A recently published systematic review of national and regional ART registry data of seven regions, including a total of 7,079,145 ART cycles, assessing the changes in clinical practice between 2004 and 2013 showed significant overall increase in the utilization of freeze thaw embryo transfer cycles [82].

The objective of elective freeze-all policy is to place the embryos in a more favourable intrauterine environment, without facing the possible adverse effects of OHSS and supraphysiologic hormonal levels over the endometrial receptivity [83]. This has been made possible by having significant advancement in the field of cryopreservation, importantly vitrification, leading to excellent post-thaw survival rate in excess of 90%.

There has been plenty of research comparing fresh vs. frozen embryo transfer in recent years. The evidence is divided with availability of studies showing similar outcome in both the groups [84, 85] or a higher pregnancy rate in the frozen ET group [86, 87]. A recent Cochrane review with four studies and 1892 couples found no superiority of fresh ET over frozen ET with moderate- to low-quality evidence [88].

61.7.6 Effect of Controlled Ovarian Stimulation (COS) on Endometrial Receptivity

Supraphysiologic levels of oestradiol and subtle increase in progesterone level during COS could lead to morphologic and biochemical alterations in the endometrium, hampering the receptivity. This leads to embryo-endometrium asynchrony leading to reduced pregnancy rates, which can be completely avoided by opting for frozen ET due to precise control of endometrial development. A recently conducted meta-analysis of three RCTs including 633 cycles showed an increase of 32% in the ongoing pregnancy rate when elective frozen embryo transfer was performed compared to fresh transfer (RR = 1.32, 95% CI = 1.10–1.59). Elective freeze-all policy with subsequent FET virtually eliminates the risk of late-onset OHSS, which tends to constitute most cases of moderate to severe OHSS during IVF. OHSS is an iatrogenic and potentially lethal complication encountered of COS in IVF. Since the major trigger for OHSS is hCG, substituting it with an agonist trigger has led to drastic reduction in the incidence of OHSS, thus improving the safety aspect of IVF. This approach together with elective freeze-all policy supports the concept of "OHSS-free clinic".

61.7.7 ET in the Presence of Uterine Anomalies

Uterine anomalies are not uncommonly reported as more and more 3D ultrasound scans are performed routinely nowadays. In a large prospective study, 13.3% of infertile females were found to have uterine anomalies with 88% being constituted by arcuate uterus [89] which is now considered as a normal variant. The pregnancy rate was not different between controls, women with arcuate uterus and women with major uterine anomalies, but the miscarriage rate was higher in the latter.

Although there is a lack of RCT evaluating the reproductive outcome of uterine anomalies undergoing IVF, controlled studies have reported increased risk of miscarriages and reduced live birth rates in women diagnosed to have uterine anomalies. Uterine anomalies are evaluated actively in women undergoing IVF since many of them would choose correction prior to ET. 3D transvaginal ultrasound is an excellent modality and is considered as gold standard for the evaluation of uterine anomalies [90].

Observational studies have suggested a beneficial effect of septal resection on IVF-ET success rate compared to those with non-resected septum [91]. In conditions like bicornuate uterus with one underdeveloped horn, cervical anatomical abnormalities, etc., the ET procedure might require necessary modifications for improving pregnancy rate like depositing the embryos in the well-developed horn in a bicornuate uterus to have a better outcome. The scenario becomes a little more complicated in the presence of cervical and vaginal septum as these need a proper speculum examination to be diagnosed and are better treated well before ET.

61.7.8 Transmyometrial ET

Transmyometrial ET (TMET), also known as the "Towako" method, has been first reported in 1993 by Kato and colleagues. This method is an effective alternative if transcervical ET is proved to be difficult due to cervical scarring or stenosis due to previous cervical surgeries. While attempts of pretreatment cervical dilatation and mock ET are done if difficult

transcervical ET is anticipated, practitioners may be faced with the difficulty in negotiating the cervical canal on the scheduled ET day with the embryos ready to transfer. Transmyometrial ET is the effective method then, if the unit has the appropriate equipment and expertise.

The equipment needed for transmyometrial ET apart from the ones required for routine ET include a vaginal ultrasound probe and a Towako ET catheter set containing a 18–19-gauge needle of 32.5 cm length and a 2.0 French catheter of 32.5 cm length.

Transvaginal-transmyometrial transfer is performed under sedation and ultrasound scan guidance. A vaginal probe is inserted, and the bevelled needle with its stylet is passed under ultrasound guidance through the anterior vaginal fornix and the myometrium of the anterior wall of the uterus. If the uterus is retroverted, the needle is passed through the posterior vaginal fornix and the posterior wall of the uterus. The needle is advanced through the myometrium to the junction with the endometrium without puncture of the latter. The stylet is then removed, and the preloaded transfer catheter is passed through the needle. After release of the embryos, the catheter is checked for any retained embryos.

The possible complications associated with the procedure include pain, junctional zone contractions with associated expulsion of embryos, haemorrhage and infection.

It has yet not become popular among reproductive medicine specialists and is seen by many as a very traumatic way to put the embryos back. The fears are pertaining to possible trauma to the endometrium and myometrium by the needle giving rise to complications including haemorrhage. Another fear is of inducing junctional zone contractions, though one study comparing TMET with very difficult transcervical ET showed that both were equally provocative in terms of junctional zone contractions.

The largest study done to date comparing these two groups showed a non-significant increase in live births in the TMET group (26.1% vs. 16.4%) and a significantly lower miscarriage rate in the TMET group (20% vs. 34%) [66]. The unquestioned need of TMET arises in case of gross anatomical defects like cervico-vaginal agenesis besides the impossible transcervical transfer due to cervical stenosis. Otherwise, there is no evidence showing the superiority of TMET over difficult transcervical ET.

61.8 Evidence-Based Recommendations for ET

- Use of ultrasound guidance for embryo transfer (Grade A)
- Removal of cervical mucus prior to ET (Grade B)
- Use of soft ET catheters (Grade A)
- Placing of the ET catheter tip in the upper or middle area of the uterine cavity, greater than 1 cm from the uterine fundus (Grade B)
- Immediate ambulation once the embryo transfer procedure is completed (Grade A)
- Immediate retransfer in case of retained embryos (Grade B)

61.9 Interventions with No Evidence of Benefit

- Acupuncture (Grade B)
- Analgesics, massage, general anaesthesia, whole systems traditional Chinese medicine (Grade C)
- Prophylactic antibiotics prior to and on the day of ET (Grade B)
- Immediate withdrawal of the embryo transfer catheter after embryo expulsion (Grade B)

61.10 Interventions Which Need Further Evidence Before Becoming a Standard of Care

- Trial transfers prior to ET
- Slow injection of embryos vs. fast injection (Grade C)

Review Questions

1. List the variables which could affect the success of embryo transfer.
2. What are the different methods of embryo loading?
3. What is the role of mock transfer in IVF-ET?
4. Which is the optimal site for placement of embryos and why?
5. What is a difficult embryo transfer and how would you overcome it?

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Bed Rest (Das)

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Catheter Soft vs firm

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G-CSF and GM-CSF: Clinical Applications in Reproductive Medicine

Wolfgang Würfel

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Learning Objectives

- Describe the colony-stimulating factors (CSFs) 2 (granulocyte-macrophage colony-stimulating factor, GM-CSF) and 3 (granulocyte colony-stimulating factor, G-CSF), the nature of their receptor interactions, and the location of these functional systems in the organism.
- Provide an overview of the physiological functions performed and supported by both CSFs, particularly with respect to reproduction.
- Describe and discuss the current state of data concerning administration of CSFs to patients with repeated implantation failure (RIF) or recurrent spontaneous abortions (RSAs).
- List issues that are still open in this respect and areas where information is still required.
- Describe potential risks of use of CSFs in reproductive medicine and during pregnancy.

Key Points

- Members of the family of colony-stimulating factors, such as granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), can be found throughout all stages of reproduction from the follicle and endometrium to the trophoblast, together with their specific receptors.
- As growth factors, G-CSF and GM-CSF have a direct positive influence on reproductive processes, such as oocyte maturation and growth of preimplantation embryos or trophoblasts, but also influence those processes indirectly by activating immunocompetent cells.
- So far, we know that when added to culture medium, GM-CSF is able to increase implantation rates in ART treatment, as is G-CSF, in the form of either intrauterine instillations or systemic administration.
- For patients experiencing recurrent spontaneous abortions (RSAs), systemic administration of G-CSF in the first trimester of pregnancy increases birth rates. Approval of G-CSF as a drug is currently undergoing examination at phase II study level.
- Administration of GM-CSF and G-CSF during ART treatments or pregnancy does not cause chromosomal aberrations in newborns; no teratogenic effects or increases in malformation rates are known.
- At the dosages and duration of administration described here, no increased risk of malignant diseases is expected.
- Questions still unanswered include issues such as when G-CSF is more beneficial than GM-CSF and vice versa, correct indications of their use, required dosages, and necessary duration of treatment.

62.1 Introduction

In human pregnancy, implantation establishes a close connection at cellular level between the mother-to-be and the embryo or fetus or – to be more precise – between the trophoblast and placenta. This connection is so close that it may even trigger the formation of fusion cells such as Langhans giant cells [1]. In pregnancy, the connection causes an intensive exchange between the two individuals, involving nutrients but also hormones, cytokines, growth factors, and cells. While these cells may already be differentiated, in many cases they are stem cells. As a result of this cell transfer, which generally takes place from the embryo/fetus to the mother-to-be, the mother-to-be becomes a chimera or, given the relatively low volumes of embryofetal cells, a "microchimera," from which the term "microchimerism" (MC) has been derived to describe the general phenomenon [2]. MC is a complex subject that is still not well understood today [3], primarily because it has two sides; the transferred stem cells appear to be capable of activating repair processes [4], yet development of autoimmune disorders may result [5]. Stem cells, which are the focus here, play a role both in MC and in placentation itself, that is, trophoblast invasion and development (cytotrophoblasts and syncytiotrophoblasts). The quality of implantation in turn determines the quality of support to the embryo/fetus and thus the further progress of pregnancy. This draws our attention to the regulatory processes that play a part here, that is, the growth factors and cytokines that control cellular growth. In recent years, members of the colony-stimulating factor (CSF) family have increasingly been shown to play a major role here [6, 7]. Five different CSFs have been identified to date, the most important of which are G-CSF (granulocyte colony-stimulating factor) and GM-CSF (granulocyte-macrophage colony-stimulating factor). They will therefore be examined in more detail.

62.2 History

In the mid-1960s, Metcalf, Sachs, and their assistants were the first to identify colony-stimulating factors for hematopoietic progenitor cells, based on cell culture assays [8]. The first factor to be isolated was G-CSF, which was shown to differentiate myelomonocytic leukemia cells in mice [9]. GM-CSF was purified in 1984 and shown to stimulate neutrophil granulocytes [10]. cDNA in mice was identified in the same year [11]. Identification of human G-CSF followed as the next step in 1985 [12, 13]. Very soon, it became evident that G-CSF and GM-CSF were two different cytokines [14, 15]. Human cDNA was likewise described in 1985 [16], while mapping of the G-CSF gene and cDNA was performed in 1986 [17].

62.3 G-CSF and GM-CSF and Their Receptors

62.3.1 Structure of G-CSF and Its Receptor

The single gene of G-CSF (CSF-3) is located on chromosome 17 (35.43–35.43) [18]. This gene has five exons with a length of approximately 2.3 kb; splicing variants are encoded in tandem at the 5′ terminus of the second intron [19]. Human glycoprotein consists of 174 amino acids and has a molecular weight of 19.6 kDa.

G-CSF acts via specific high-affinity receptors. Basically, it was thought that there were four different receptors. However, there is one encoded receptor, also known as cluster of differentiation (CD) 114. The CSFR gene is located on chromosome 1 (36.47–36.48) [20]; its molecular weight is approximately 19.06 kDa.

The G-CSF receptor belongs to the cytokine receptor superfamily and performs its functions by means of tyrosine phosphorylation and activation of members of the Janus protein kinase (Jak) family. Additionally, an activation pathway of the signal transducers and activators of transcription (STAT) family is described.

62.3.2 Structure of GM-CSF and Its Receptor

Although G-CSF and GM-CSF are extremely similar in some respects, the gene for GM-CSF (CSF-2) is located on a different chromosome, namely, chromosome 5 (132.07–132.08). Although DNA codes for 144 amino acids, the active form of GM-CSF consists of only 127 amino acids. Its molecular weight is thus no longer 23 kDa, but 16.3 kDa [21].

The GM-CSF receptor is a heterodimer consisting of one α- and one β-chain [22]. The α-chain is designated CSF2RA (CDw 116) and is coded for the pseudoautosomal region of the X and Y chromosomes (Xp 22.32). The β-chain (CSFRB) is located on chromosome 22 (12.2–13.1) [23].

The GM-CSF receptor is likewise part of the type I cytokine receptor superfamily. Like the G-CSF receptor, it performs its functions via members of the Jak or STAT family [22].

62.3.3 Tissue Distribution of the G-CSF Receptor and the GM-CSF Receptor

G-CSF and GM-CSF receptors can be detected in many myeloproliferative tissues and their cells, for example, in monocytic cells like T-cells, natural killer (NK) cells, macrophages [24, 25], and even platelets [26].

The following description will be limited to an examination of the receptors in connection with reproductive functions.

The GM-CSF receptor is found in bovine ovarian follicles and granulosa cells [27] and can be identified in human corpora lutea throughout the luteal phase [28]. However, expression of the subunits appears to vary, at least in mice [29]. Expression in the endometrium is at its highest during the mid-secretory phase of the endometrium [30]; after menopause, expression drastically declines [31].

G-CSF receptor expression increases during maturation of the preovulatory follicle [32]; expression is also proven in the second part of the cycle, that is, luteinized human granulosa cells [33]. In human endometrial cells, G-CSF receptor expression exists throughout the entire cycle [34]; expression is under the positive influence of estrogens [35].

The GM-CSF receptor can be identified in both bovine and human testicular tissue, where its activation results in improved sperm motility [36].

The G-CSF receptor is expressed on the trophoblast [37] and in the placenta [38]. The highest G-CSF receptor expression can be seen in the first trimester. It declines over the second trimester and rises again in the third trimester [39]. The G-CSF receptor is easily identified in the human extravillous trophoblast during the first trimester [40] as well as in other trophoblast cells [30].

In the human fetus, G-CSF and GM-CSF receptors are present in nearly all tissue types, for example, hematopoietic and neural stem cells [41, 42] and cells of the kidneys and the gastrointestinal tract [43, 44], and in various areas of the brain and central nervous system [45]. They can first be identified 8–16 weeks after conception [41]. Adults also show both receptors in virtually all types of body tissue.

62.3.4 Cellular Synthesis of G-CSF and GM-CSF

G-CSF and GM-CSF are synthesized by NK cells, particularly uterine NK (uNK) cells (CD 56^{bright}) [46, 47]. Further immunocompetent cells with synthesis of both colony-stimulating factors include macrophages/monocytes [48], T-cells [49], and dendritic cells [50].

G-CSF and GM-CSF are synthesized by granulosa cells and luteal cells [33, 51], increasing particularly during the preovulatory phase [32]. This synthesis can be activated by estrogens [35] and progesterone and, interestingly, by components of seminal fluid [52].

G-CSF is synthesized in decidual cells [53] and in the chorionic villous trophoblast [54], but not in the cytotrophoblast. The synthesis rate for both cell types is significantly higher in the first trimester than at term. Both trophoblast and decidual syntheses have been shown for GM-CSF [30], although it should be remembered that all these tissue types are highly permeated by immunocompetent cells, chiefly large granular leucocytes (LGL) (CD 56 ^{bright}), and may potentially secrete G-CSF and GM-CSF.

G-CSF and GM-CSF can be identified in amniotic fluid [55, 56]. Their concentration appears to increase in

pathological pregnancies, primarily with inflammatory complications such as amniotic infection syndrome (AIS) [57], concomitant with increasing receptor expression in the placenta [58].

62.4 Physiological Significance of G-CSF and of GM-CSF

G-CSF is considered to be a major cofactor during the ovulation process in humans [58].

GM-CSF promotes follicular development (in rats) [59] and cumulus cell expansion in cattle [60].

Thickening of the endometrium in rats is induced by G-CSF [61]. In diabetic rats (experimental diabetes mellitus), intraperitoneal injection of G-CSF significantly reduced degeneration of ovarian follicles and the decline of AMH (anti-Müllerian hormone) [62].

A higher percentage of hatching blastocysts in mice can be seen under the influence of G-CSF and GM-CSF [63]. In 2005, GM-CSF was shown to have the same effect on human blastocysts, with positive influence on both generation rate and growth [64]. GM-CSF promotes glucose uptake and results in enhanced proliferation and/or viability of blastomeres in murine blastocysts [65]. G-CSF-deficient mice are viable and able to have offspring, but have lower fertility rate and a significant higher rate of spontaneous abortions [66]. G-CSF works in an anti-abortive manner in mice [67].

Both GM-CSF and CSF-1 are capable of accelerating differentiation of the human trophoblast. This is accompanied by an increase in the synthesis rate of human placental lactogen (hPL) and human chorionic gonadotropin (hCG) [68].

G-CSF is able to pass the placenta barrier in rats [69]. This has also been demonstrated in mice, where increased concentration in the fetus can be seen only 30 min after a single dose of 50 ug/kg rh-G-CSF (with peak concentration after 2 h) [70]. The ability of GM-CSF to pass the placenta barrier has also been proved [71].

Chorial cells start proliferating under the influence of G-CSF [53]. During pregnancy, concentrations are higher than in nonpregnant women [72], although this is not undisputed [70].

G-CSF and GM-CSF are able to enhance concentrations of regulatory T-cells [73] and to activate dendritic cells [74]. G-CSF is also able to reduce toxicity of NK cells [75].

G-CSF and GM-CSF lead to increased migration and proliferation of endothelial cells [76].

In summary, it can be established that both G-CSF and GM-CSF (together with CSF-1, i.e., macrophage colony-stimulating factor (M-CSF)) and their receptors are located in many different areas of the reproductive tract and are expressed in many different stages of the reproductive process, in some cases with sustained effects [77]. Absence of these factors or their reduced expression leads to lower fertility per se, not only in animal testing, and to an increase in pregnancy pathologies [64, 78].

62.5 Applications in Diagnosis

ART treatments such as IVF or ICSI appear to show a positive correlation between concentrations of G-CSF in follicular fluid and the probability of implantation or subsequent pregnancy [79–85]. The publications of this working group indicate that this only applies to G-CSF, with concentrations >30 pg/ml offering the highest probability of subsequent implantation of the embryo (class I) and concentrations of <18.4 pg/ml (class III) the lowest; while concentrations did not necessarily correlate to embryo morphology, the highest implantation rates were shown by embryos with excellent morphology and extremely high follicular G-CSF concentration. Interestingly, this correlation clearly also applies in the case of patients with pronounced ovarian insufficiency (AMH < 0.1 ng/ml).

A testing system for these concentrations is available commercially under the name of Diafert™.

The approach is extremely interesting with respect to improving the accuracy of estimated individual prognosis [86], but also of prognosis with respect to individual oocytes; admittedly, however, application of the system in practice in follicular puncture is extremely complex.

62.6 Clinical Applications

Today, recombinant (genetically engineered) substances are commonplace in treatment of humans. In the case of GM-CSF, these may be molgramostim or sargramostim, the latter produced from yeast cells. Filgrastim is a recombinant human G-CSF analog from *E. coli*, while lenograstim is produced from CHO cells and is still glycosylated in position 133, that is, somewhat more physiological. PEGylated forms are approved as lipegfilgrastim and pegfilgrastim.

However, it should be noted that many countries currently only allow h-CSF analogs, but not h-GM-CSF analogs.

62.6.1 G-CSF and Oocyte Maturation

A positive effect on ovulation induction is also noted; a single dose of rh-G-CSF (lenograstim, 100 mg) 48 h before administration of hCG results in a significant reduction of LUF (luteinized unruptured follicle) syndrome in CC (clomifene citrate) cycles [87], which may enable approximately 90% of all LUF syndromes to be avoided [88].

62.6.2 G-CSF for Thin Endometrium

In 2011, Gleicher et al. [89] first reported on intrauterine instillations in four patients with thin endometrium as part of an ART program. After G-CSF instillation, all four patients showed increased endometrial volume, and all four underwent embryo transfer (ET) resulting in pregnancy. A subsequent noncontrolled cohort study confirmed the initial

positive impression of G-CSF instillation [90]. Two further studies showed a positive effect on endometrial growth (unresponsive thin endometrium) and increased pregnancy rate [91, 92]. Tests with rats furnished proof that this phenomenon is actually based on the increased regeneration and proliferation of endometrial cells [93].

In contrast, after G-CSF instillation in patients with thin endometrium, Eftekar et al. [94] noted higher implantation and pregnancy rates but no increase in endometrial thickness.

Of course, instillation appears to provide no benefits for "normal" ART patients with well-developed endometrium, neither increasing endometrial thickness nor boosting pregnancy rates [95, 96]. (We have ourselves observed sustained endometrial growth through systemic administration of rh-G-GSF (lenograstim, 13 m) every second or third day during the follicular or stimulation phase.)

With respect to GM-CSF, no data on intrauterine instillations with ART patients have yet been published.

In summary, intrauterine G-CSF instillations are clearly able to improve implantation rates after embryo transfer for patients with implantation problems and poorly developed endometrium; however, they are ineffective in patients with normal endometrium. Similar results are anticipated from GM-CSF.

However, endometrial thickness is not a very reliable parameter. It would be more important to know the type of cells which make up the endometrium in patients with good and poor implantation outcomes; key roles are likely to be played by uterine NK cells, but also peripheral cells. This information would enable more precision in establishing indications.

There is also uncertainty over applicable dosages, particularly in correlation with endometrial findings.

62.6.3 G-CSF and GM-CSF in Culture Media of ART Cycles

As described here, GM-CSF results in an increased blastocyst generation rate and accelerated growth, shown in tests including GM-CSF-deficient knockout mice [97]. The effect was also shown in human preimplantation embryos where 2 ng/ml of rh-GM-CSF was added to the culture medium for ART treatment [98]. Furthermore, GM-CSF appears to be capable of preventing inner cell mass apoptosis [99]. A randomized double-blind placebo-controlled multicenter study [100] showed that supplementation of culture medium by adding rh-GM-CSF significantly raises pregnancy and birth rates; out of 1149 embryo transfers recorded, the implantation rate rose significantly from 20% to 23.5%, and the birth rate rose from 24.1% to 28.9%. Patients with a previous history of one or more abortions benefited particularly from GM-CSF.

The culture medium is now commercially available under the name EmbryoGen™. It is approved for medical histories involving a minimum of one spontaneous abortion. Under the name of BlastGen™, the medium is now available for 5-day cultures in addition to 3 days.

A culture medium with added 0.5 ng rh-G-CSF/ml is also a patent applied for [101].

62.6.4 Systemic Application of h-GM-CSF and/or h-G-CSF in Patients with Repetitive Implantation Failures (RIFs) in an ART Program

There is now no doubt that some patients show implantation problems. Plentiful evidence is available from ART treatments, in cases where pregnancy does not result despite multiple embryo transfers under highly promising conditions (e.g., young age of patient, excellent embryo morphology, blastocysts where applicable).

As early as 2000, we reported [102] on our experiences with a single dose of GM-CSF (300 ug molgramostim) or G-CSF (34 m filgrastim) at the time of embryo transfer. Results are shown in ◘ Table 62.1. While filgrastim was very well tolerated, some cases showed undesirable side effects after administration of human-derived GM-CSF, such as high blood pressure and chest tightness.

◘ **Table 62.1** Results of the pilot study for RIF patients [102]

	Molgramostim 300 ug	No medication	Filgrastim 34 m	No medication
Number of patients	107	107	69	69
Number of embryo transfers (day 2)	107	106	69	69
Clinical pregnancies	46	21	35	13
Pregnancy rate/embryo transfer (clinical pregnancies)	42.9%	19.8%	50.7%	19.8%
Abortions (abortion rate)	9 (19.5%)	2 (19.0%)	7 (29%)	2 (15.4%)

Definition of RIF: More than four treatment cycles with at least eight embryos of good morphology transferred. ET was performed on day 2. The age of all groups was between 37 and 38

Patients in a randomized controlled double-blind study (RCT) from 2016 likewise received 300 ug G-CSF as a single shot at the time of ET [103]. The study showed an increase in (clinical) pregnancy rate from 14.3% to 37.5%. The same procedure was reported by Davari-tanha [104], who noted a significant rise in the implantation rate, albeit without affecting the rate of spontaneous abortion.

In 2010, we reported on continuous application of lenograstim (13 m every third day after embryo transfer) to RIF patients [105]. To obtain a more precise purely clinical indication of RIF, we identified any deficiency in activating killer immunoglobulin-like receptors (KIRs) in advance [106, 107]. Application after embryo transfer was restricted to cases with this deficiency, combined with a predefined RIF constellation. Compared with the average pregnancy rates of our ART program, rates with G-CSF rose significantly, with a pregnancy rate of 42% per ET on day 2 and 73.8% on day 5. An ongoing observational study (>800 embryo transfers) showed lower figures but continued to record significant higher results with administration of G-CSF, particularly for embryo transfers on day 5. Gil-Herrera [108] pursued the same strategy with a small group of patients and reported high pregnancy rates, low spontaneous abortion rates, and excellent tolerance of SC injections.

In 2012, Scarpellini and Sbracia [109] published a randomized controlled study of 109 RIF patients. ET was performed on day 2 after ET patients had been given a daily dose of 60 mg rh-G-CSF. In cases of positive pregnancy tests, this regimen was continued for a further 40 days. The treatment group showed a (clinical) pregnancy rate of 43.1% per ET, compared to 21.6% in the placebo group (saline injections), resulting in a highly significant difference ($P < 0.001$).

In summary, it can be assumed that G-CSF, in the form of both single shot and continuous administration, is capable of improving implantation and pregnancy rates in RIF patients [110–112]. However, with the exception of the clinical definition of RIF, there are still no clear-cut diagnostic criteria for identifying patients that would benefit from G-CSF. If these criteria existed, patients could be identified at primary level without needing to go through a series of unsuccessful treatments.

A plausible initial approach in this direction involves identification of KI receptors in correlation with the HLA groups of both partners. There are doubtless constellations in which embryo-maternal communication is predictably disrupted [106, 107], so that a central growth factor like G-CSF (or GM-CSF) can stabilize pregnancy.

A further open question is the issue of dosages, although these are similar throughout all available studies. No study designed to identify dosage has been conducted to date.

No further published data are available with respect to GM-CSF. This may be because virtually no specialty pharmaceuticals are available; however, a patent application for this indication is pending with the European Patent Office (EPO) [113].

62.6.5 G-CSF in Patients with Recurrent Spontaneous Abortions (RSAs)

Data from the Severe Chronic Neutropenia International Registry (SCNIR) gave the first hints that CSFs may be able to lower the abortion rate. For example, Boxer et al. reported in 2010 [114] that patients suffering from neutropenia and receiving G-CSF during pregnancy showed significantly fewer abortions, namely, 5%, compared to 32% in patients who did not receive G-CSF. At the same time, premature births and infant mortality were observed to decline.

In 2009, Scarpellini and Sbracia [115] reported the results of a randomized placebo-controlled study of RSA patients. The main results are depicted in ◘ Table 62.2.

The birth rate in the treatment group was significantly higher compared to the placebo group. The abortion rate and the obstetric outcome for both groups were similar; no malformations were reported. Striking results are the significantly increased hCG levels in the G-CSF group, which is approximately 30% higher than in the control group (9th week of gestation).

Santjohanser et al. in 2011 [116] reported on RSA patients in an ART program (◘ Table 62.3). The report showed that the G-CSF group experienced higher pregnancy rates per embryo transfer and lower spontaneous abortion rate in subsequent pregnancies. This difference was also noted in comparison to the third patient group, which received drugs such as heparin,

◘ **Table 62.2** Main results of the study by Scarpellini and Sbracia [115] in patients with recurrent spontaneous abortion (RSAs) receiving G-CSF vs. placebo

	Rh-G-CSF	Placebo	*P*-value
Patients	35	33	
Age (years) (start of pregnancy)	34.9 ± 2.9	33.8 ± 2.9	
BMI (start of pregnancy)	27.4 ± 1.9	33.8 ± 1.8	
Smokers (>10 cigarettes/day)	1	2	
Previous miscarriages	5.5 ± 0.4	5.6 ± 0.3	
Gestational week of miscarriage	6.1 ± 1.2	6.4 ± 1.1	
Live births (%)	29 (82.8%)	16 (48.5%)	0.0061
Renewed miscarriages (%)	6 (17.2%)	17 (51.5%)	0.0061
Gestational week of miscarriage (mean ± SD)	6.0 ± 1.1	6.2 ± 1.0	0.6989
Weight of newborns (g) (mean ± SD)	3050 g ± 220 g	3125 g ± 240 g	0.3098

Table 62.3 ART patients with recurrent miscarriages (RSA). Main results of the study of Santjohanser et al. [116]

	Filgrastim	No medication	Other medications (e.g., low-molecular-weight heparin, ASS (100 mg), prednisone/dexamethasone, doxycycline)
Age (years)	37.6 ± 4.0	37.6 ± 4.4	37.6 ± 4.4
Previous ART cycles	6.5	5.3	6.0
Oocytes	9.4 ± 5.6	9.6 ± 5.0	7.8 ± 4.4
Fertilized oocytes (pronuclear stages)	5.7 ± 3.9	5.0 ± 3.4	4.9 ± 3.4
Transferred embryos	2.1	2.5	2.4
Anamnestic early miscarriages	2.7 ± 1.3	0.9 ± 0.9	0.6 ± 0.7
Anamnestic late miscarriages	0.2 ± 0	1.8 ± 1.0	1.8 ± 1.0
Embryo transfers (ETs)	49	33	45
Pregnancies per ET (%)	23 (46.9%)	8 (24.4%)	12 (26.6%)
Clinical abortions (%)	7 (30.4%)	4 (50%)	6 (50%)

cortisone, ASS, and/or immunoglobulins, that is, substances that are, or should be, designed to improve the pregnancy rate per ET and decrease spontaneous abortion rates.

In 2015, we published our data [117] available to date concerning treatment of RSA (and RIF) patients who received doses of 2 × 13 m rh-G-CSF per week up to complete autonomy of the trophoblast/placenta, that is, up to the 12th week of pregnancy. The data captured over 600 pregnancies. Data on the progress of pregnancy were available in 88% (RIF) and 91.8% (RSA) of cases, respectively. The baby take-home rate per pregnancy begun was 72.4% and 78.1%, respectively; around 86% of births were after the 37th week of pregnancy. In cases of repeated abortion, we always tried to conduct genetic analysis of the abortion material. Where this was possible, genetic abnormality rates of 67.4% and 60.9%, respectively, were found. Medication was without documented undesirable side effects in all cases; accompanying nausea (morning sickness) was virtually universal. Strikingly, all patients in our care showed high levels of hCG, progesterone, and estradiol. Sonograms performed between the 8th and 11th week of pregnancy on RIF patients with known embryo transfer dates often showed preemptive biometric data. No increase was seen in documented malformations compared to the normal population; in fact, the percentage trend was actually lower than the average.

Now, in early 2017, we have data from over 900 pregnancies; figures and observations have shown no major changes.

These studies and observations show without a doubt that "G-CSF deficiency syndrome" or "CSF deficiency syndrome," that is, "CSF-DS," must exist. However, this is based less on an absolute deficiency in CSFs (which would cause serious illness in patients) than on reduced release of these substances during pregnancy and in utero in particular, in other words, a communication problem.

We assume that uNK cells, in particular with their killer immunoglobulin-like receptors (KIRs), are in close dialog with the trophoblast, that is, the conceptus [118, 119]; if this dialog is interrupted, disruption to implantation and growth of the embryo results. This is the case, for example, where activating KI receptors are absent and the partners' HLA groups correspond to a large extent, particularly the HLA-C groups that are so important for communication (or have only weak effects: HLA-C groups 2) [106, 107, 120]. Systemic administration of G-CSF is then likely to be useful in compensating for this relative deficit and stabilizing the pregnancy. The positive effect on growth (particularly of the trophoblast) can easily be seen in the increased hCG values. Accelerated growth of the trophoblast automatically leads to increased transfer of embryo cells to the maternal organism, which neatly explains the virtually universal nausea experienced in pregnancy (microchimerism; see above).

No data are available for systemic administration of GM-CSF to RSA patients. This is surprising, given that EmbryoGen™/BlastGen™ culture media are approved for cases of previous spontaneous abortion after ART treatment. It would thus be only logical to support the embryo not only up to the time of transfer but also beyond it, that is, during and after implantation.

The effects of G-CSF and GM-CSF on embryo growth, implantation, and pregnancy as described above would naturally suggest their use in treating further types of pregnancy pathologies. Patients have already been granted for administration of G-CSF in the treatment of preeclampsia [121] and for prevention of premature birth [122].

Nora Therapeutics, the holder of these patients, is currently driving G-CSF approval to avoid RSA (phase II study). Scarpellini and Sbracia have already patented (with the EPO) a procedure – as already mentioned – for administration of GM-CSF for the same indication [113].

62.7 Risks

62.7.1 G-CSF and GM-CSF Before and During Pregnancy

Where EmbryoGen™/BlastGen™ are used, embryos are "bathed" in a culture medium with added 2 ng/ml rh-GM-CSF for several days, 24 h a day. Agerholm et al. showed as early as 2010 [123] that this procedure neither positively nor negatively modifies the chromosomal constitution of preimplantation embryos.

SCNIR holds relatively extensive data on the administration of G-CSF during pregnancy. No higher morbidity or mortality was observed [124, 125]. Boxer et al. [114] observed fewer severe maternal and neonatal complications and no significant increase in the rate of malformations. This is also the view of the European branch of SCNIR, so that women with chronic neutropenia are no longer advised to avoid G-CSF treatment in pregnancy. This is confirmed by several case reports of women who gave birth to healthy babies after having received G-CSF injections [126, 127].

In addition, there are numerous reports of pregnant women who have received G-CSF, either to support chemotherapy [128] or to mobilize autologous stem cells during chemotherapy [129–132]. No teratogenic effects or malformations were seen.

With regard to peripheral blood stem cell (PBMC) harvesting during pregnancy, and therefore during administration of G-CSF, on the basis of currently available information, a review [133] came to the conclusion that treatment using G-CSF is safe in pregnancy and that healthy pregnant women can donate PBSC and bone marrow after receiving G-CSF.

As described above, according to our information, administration of G-CSF in pregnancy does not result in increased risk of malformation in live births, in fact, rather the opposite. A tendency toward higher birth weights has been noticed.

62.7.2 Risk of Induction of Malignant Cells

Chemotherapy, that is, the treatment of malignomas, is the "classical" indication for G-CSF/GM-CSF. Current guidelines see no enhanced risk of lymphoproliferative disorders, tumor progression, or induction of a second malignoma (e.g., guidelines of EORTC and ASCO [134]; of ESMO [135]; of DGGG and DKG, 2012, for breast cancer [136]; and of DKG, 2013, for Hodgkin's lymphoma) [137]. Additionally, G-CSF has been proved not to increase the percentage of circulating tumor cells (CTC) [138, 139].

Healthy bone marrow donors receiving filgrastim for a short period are not at increased risk of developing myeloid leukemia and/or myelodysplasia (observation period: 3–6 years) [140], similarly stated by the National Marrow Donor Program Registry [141].

However, in some patients, continuous high-dosage administration over several years may result in development of myelodysplasia syndrome (MDS) or acute myeloid leukemia (AML). The risk is estimated by SCNIR at 9% in 13 years [142] and by Rosenberg at 2.3% after 10 years [143].

62.8 Summary

Historically, representatives of the family of colony-stimulating factors (CSFs), such as granulocyte colony-stimulating factor (G-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF), were long assumed to be cytokines for regulating myeloproliferative processes, as their names imply. It is now known that they are, in fact, ubiquitously occurring and ubiquitously acting growth factors which are shown to exist at practically all levels of reproduction, together with their specific receptors. For example, they play a role in regulating oocyte maturation and in spermiogenesis, as well as in the ovulation process; they are present in the endometrium, accelerate the generation and growth of preimplantation embryos, and support the implantation process and trophoblast growth. Accordingly, they are already in clinical use. Concentration of these substances in the follicles correlates with later implantation rates of the resulting fertilized oocytes, which can be used in diagnosis. Addition of GM-CSF to the culture medium used in ART treatment increases the percentage of blastocyst formation and growth, as well as subsequent pregnancy and growth rates. The same clearly applies to G-CSF, be it used as intrauterine instillations or systemic administration. Patients with repetitive implantation failure (RIF) or a history of abortion appear to benefit from the treatment. The same evidently applies to patients with spontaneous conception but with recurrent spontaneous abortions (RSAs). In these cases, systemic administration of G-CSF likewise results in a significantly higher birth rate. However, indications for the specific use of G-CSF or GM-CSF are as yet unclear. HLA typing of both partners in combination with determination of KIR genes is likely to be useful in this respect. Further open questions concern dosage levels for systemic administration and the duration of administration. Available recombinant specialty pharmaceuticals are well tolerated, with no indication of teratogenic effects, chromosomal changes, or even increased malformation rates among live births. In addition, at the dosages and duration of administration used here, induction of malignancies is not anticipated. It may be assumed that CSFs will continue to be used in other indications in the future, such as prevention of premature birth or preeclampsia.

Summary Bullet Points

- GM-CSF and G-CSF play an important role in reproductive functions, not only in regulation of myeloproliferative processes.
- In diagnostic terms, determination of G-CSF in follicular fluid is already used to predict later likelihood of pregnancy in ART treatment.
- Addition of GM-CSF to the culture medium used in ART treatment improves implantation and birth rates,

particularly for patients with repeated implantation failure (RIF) or history of abortion.
- Intrauterine instillation or systemic administration of G-CSF is evidently also able to increase implantation and birth rates resulting from ART treatment.
- Systemic administration of G-CSF to patients with recurrent spontaneous abortions (RSAs) increases the birth rate.
- No teratogenic effects or increased malformation rate is known to result from administration of GM-CSF or G-CSF; at the dosages described, induction of malignancies is not anticipated.
- Indications for the specific use of G-CSF or GM-CSF are as yet unclear, as are more precise indications and which dosage levels and duration are the most favorable in systemic administration.

Review Questions

1. Is the role of G-CSF and GM-CSF limited to regulation of myeloproliferative processes?
2. Does the concentration of G-CSF in follicular fluid have any predictive significance?
3. Can the presence of GM-CSF in the culture medium increase pregnancy rates in ART programs?
4. What are the effects of intrauterine instillation of G-CSF in an ART program?
5. Can systemic administration of G-CSF increase birth rates among patients with recurrent spontaneous abortions?
6. Does the administration of G-CSF or GM-CSF involve risks, for example, of malformation or malignoma?
7. What are the indications for use of G-CSF and GM-CSF?

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The Freeze-All Cycle: A New Paradigm Shift in ART

Bruce S. Shapiro, Forest C. Garner, and Martha Aguirre

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Learning Objectives

- The history of in vitro fertilization (IVF) paradigm shifts and how embryo/oocyte cryopreservation and the freeze-all strategy fit into that history
- The evidence regarding success rates of the freeze-all strategy in selected patient populations
- The evidence regarding maternal and perinatal risks relevant to fresh or frozen embryo transfer
- Pertinent new research areas

63.1 The Classic IVF Paradigm

63.1.1 A Brief History of IVF Technology

It is well known that the first live birth of an infant following in vitro fertilization (IVF) was reported by Steptoe and Edwards in 1978 [1]. That first live birth followed a fresh autologous embryo transfer in a patient with tubal factor infertility. However, that was neither their first embryo transfer nor their first reported pregnancy. Their first reported pregnancy was announced 2 years before the famed live birth [2]. That pregnancy was ectopic, a result that they attributed to the untoward effects of controlled ovarian stimulation (COS). In 1977, before the first live birth from IVF, Edwards and Steptoe discussed the advantages of avoiding the negative effects of uterine COS exposure by freezing all embryos for subsequent frozen-thawed embryo transfers (FETs) of single embryos. In that discussion, they attributed previous failures to an inability to control "the endocrine conditions necessary for implantation" and attributed implantation failures to "abnormal endocrine conditions arising in patients treated with HMG and HCG" [3].

Since then, the technology has improved greatly, and the indications for IVF are now numerous. Despite the early reservations regarding the effects of COS on the uterine environment, the fresh autologous cycle remained the dominant type of IVF cycle for more than three decades. However, recent technological improvements in cryopreservation techniques along with a series of findings proving untoward effects of COS on endometrial receptivity, maternal risk, and perinatal risk have challenged the fresh cycle's dominance in favor of a freeze-all strategy. In a freeze-all cycle, all embryos or oocytes are cryopreserved for thaw and transfer in a subsequent cycle without uterine COS exposure.

In the classic fresh autologous cycle, the patient receives injections of exogenous gonadotropins, typically recombinant follicle-stimulating hormone (rFSH) and/or urinary-derived human menopausal gonadotropin (hMG), so that her ovaries may develop multiple follicles that will yield multiple mature oocytes. As the follicles mature, rising follicular production of estradiol and, later, progesterone can modify hypothalamic behavior, so that hypothalamic gonadotropin-releasing hormone (GnRH) can cause pituitary release of a surge of luteinizing hormone (LH). A premature LH surge can greatly reduce the chance of success, often requiring the patient to repeat a cycle of COS. Therefore, from the late 1980s, prolonged daily administration of GnRH agonist was used to desensitize the pituitary to GnRH and therefore prevent premature LH surges. By that time, cleavage-stage embryo transfer was the norm, typically on day 3 of embryo development, and embryos were morphologically selected based on cell count, symmetry, and fragmentation.

The use of the agonist for pituitary downregulation limited the options for final oocyte maturation to human chorionic gonadotropin (hCG), resulting in a significant frequency of ovarian hyperstimulation syndrome (OHSS) in patients with high ovarian response to gonadotropin stimulation.

Unrecognized at the time, exposure to COS with exogenous gonadotropins resulted in advanced endometrial development and impaired endometrial receptivity via embryo-endometrium asynchrony, reducing the ability of the transferred embryos to implant and also negatively affecting the health of resulting offspring [4]. The low implantation rates in the 1990s, often <20%, compelled clinicians to compensate by transferring many embryos into each patient's uterus, risking multiple pregnancy with corresponding additional risks to the health of offspring.

Routine extended culture to the blastocyst stage was adopted by some centers in the late 1990s and has steadily become more common. Extended culture eliminates from consideration those embryos that cannot form blastocysts. Also, blastocyst morphology is more complex than cleavage-stage morphology, allowing more advanced grading of embryo quality and therefore potentially better embryo selection. Implantation rates are therefore greater with blastocyst transfer than with cleavage-stage transfer, supporting the transfer of fewer embryos and wider use of elective single-embryo transfer (eSET).

The classic IVF cycle changed slightly but significantly at the end of the twentieth century, when GnRH antagonists were approved by the US Food and Drug Administration (USFDA). The use of a GnRH antagonist allowed the agonist to be reserved for possible use as an ovulatory trigger, causing final oocyte maturation by inducing a surge of pituitary LH. Agonist trigger virtually eliminated the risk of clinically significant OHSS [5], although published case reports prove that rare cases of clinically significant OHSS remain possible. The daily GnRH antagonist injections were usually initiated late enough in the cycle to allow estrogen receptor modulators or aromatase inhibitors to be used early in the cycle to stimulate hypothalamic GnRH release, so that pituitary FSH could augment exogenous FSH for several days.

The classic IVF paradigm remained essentially static since the end of the twentieth century. As this is written, some centers in the United States still routinely use agonist downregulation, hCG trigger, and fresh transfer of multiple cleavage-stage embryos, practices associated with reduced implantation rates and increased health risks to patients and offspring when compared to modern practice.

63.1.2 The Fresh Embryo Transfer

Live birth rates following fresh transfer increased steadily until approximately 2008 [6], after which there was little or no improvement (Fig. 63.1). With FET, the corresponding improvement was more rapid and continued until at least 2014, the latest data available (Fig. 63.2), so that live birth rates per transfer with FET exceeded those with fresh transfer in each age group excepting only the youngest (<35 years) age group.

Implantation rates with fresh transfer continued to increase in recent years (Fig. 63.3), but the corresponding increases in implantation rates following FET (Fig. 63.4) were more rapid, and implantation rates with FET now exceed those of fresh transfer in every age group.

Zooming in on one age group for illustration (Fig. 63.5), in patients aged 38–40, national average live birth rates following fresh transfer were 26.5% in 2004, increased to 28.2% in 2008, and then remained essentially constant, with a value of 28.0% in 2014. The risk ratio (RR) for live birth was 1.06 when comparing 2014 to 2004. The corresponding birth rates for FET were 23.1%, 26.1%, and 38.3%, for a RR of 1.66 over the same period. As of 2014, the latest data

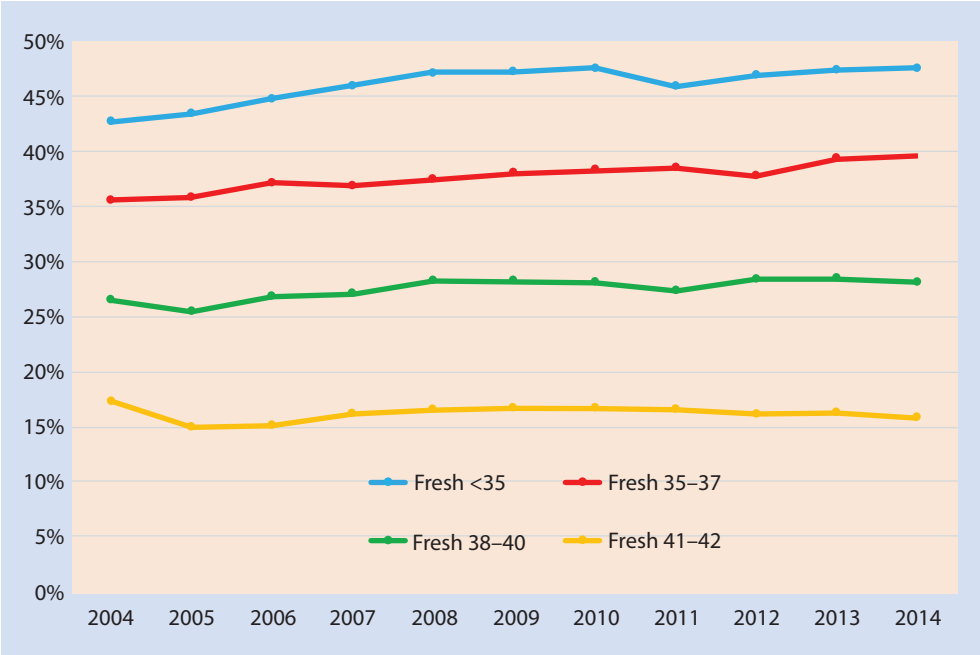

Fig. 63.1 Trends in national average live birth rate per fresh autologous transfer from reporting year 2004 thru reporting year 2014 as reported by the CDC, broken out by age group. Note moderate increasing trends prior to 2008, with little or no improvement thereafter

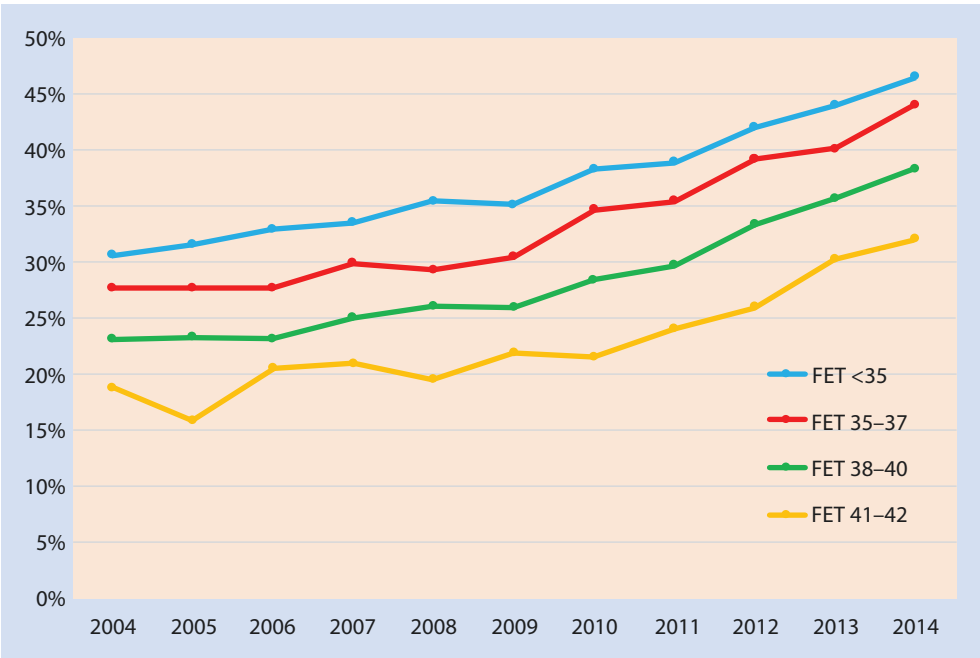

Fig. 63.2 Trends in national average live birth rate per autologous FET from reporting year 2004 thru reporting year 2014 as reported by the CDC, broken out by age group. Note generally increasing trends throughout the period. Compare to Fig. 63.1

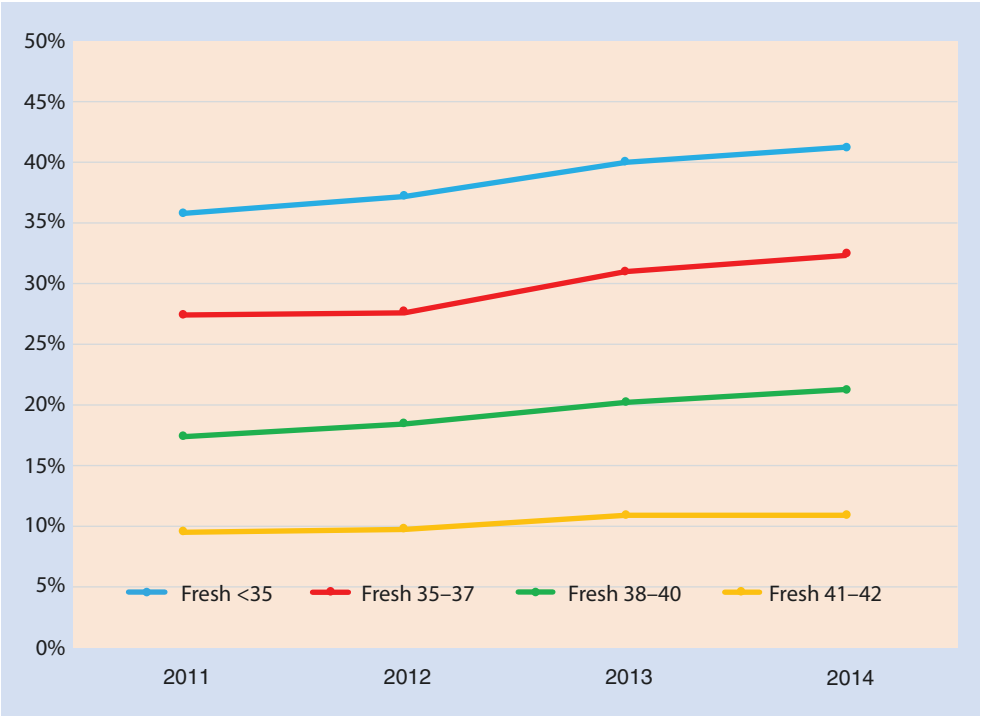

Fig. 63.3 Trends in national average implantation rate per transferred embryo in fresh autologous transfers from reporting year 2011 thru reporting year 2014 as reported by the CDC, broken out by age group. Note moderate increasing trends throughout the period

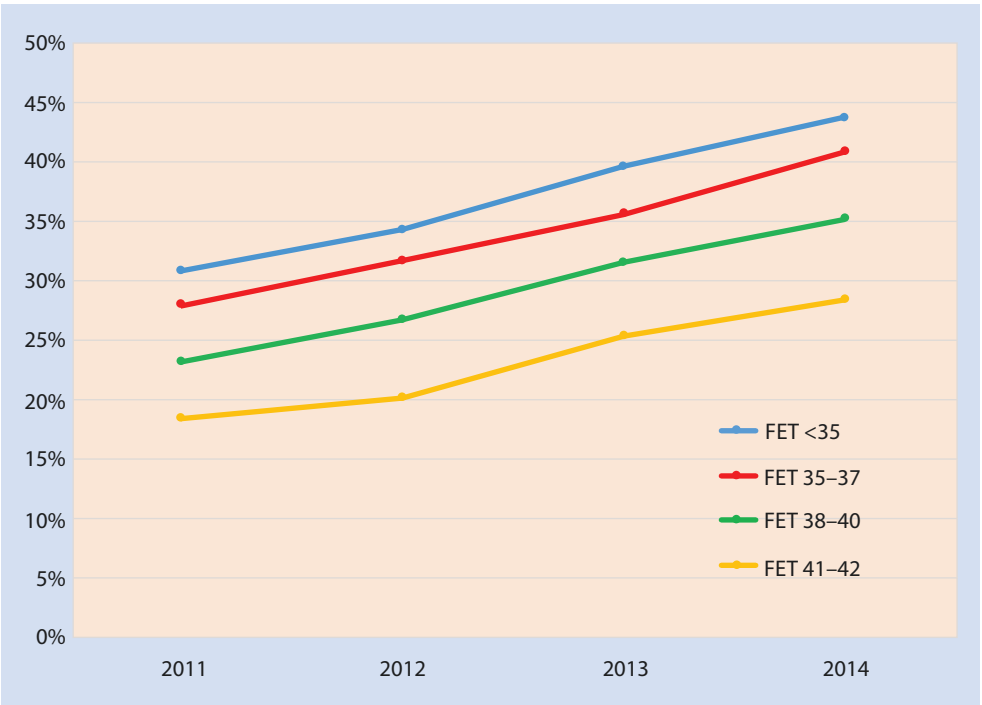

Fig. 63.4 Trends in national average implantation rate per transferred embryo in autologous FET from reporting year 2011 thru reporting year 2014 as reported by the CDC, broken out by age group. Note increasing trends throughout the period. This time period was selected because the CDC started reporting implantation rates for FET in 2011. Compare to Fig. 63.3

available, the birth rate following FET exceeded that of fresh transfer by a risk ratio of 1.37. The Centers for Disease Control and Prevention (CDC) published implantation rates for FET starting in 2011, and Fig. 63.5 also shows that implantation rates following FET increased more steeply than those following fresh transfer in 2011–2014.

The increasing success rates with FET probably reflect improving cryopreservation techniques, increasing use of freeze-all cycles so that more primary (best-of-cohort, rather than supernumerary) embryos are used in FET, and, in the older age groups, perhaps increasing use of preimplantation genetic screening (PGS) of embryos.

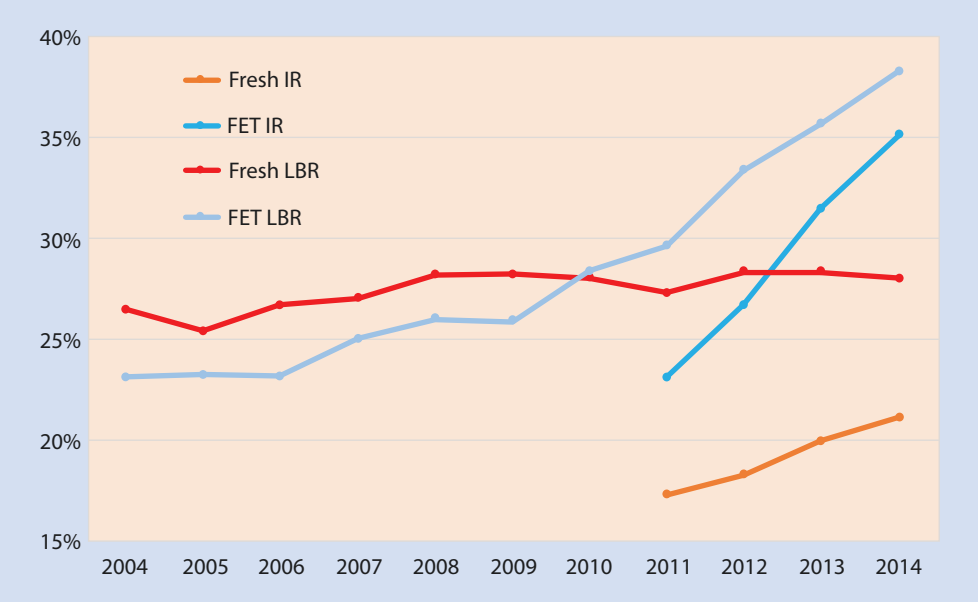

Fig. 63.5 Trends in national average live birth rate (LBR) and implantation rate (IR) per transferred embryo in autologous transfers from 2004 thru 2014 as reported by the CDC for patients aged 38–40, broken out by cycle type. Note the more rapidly increasing trends for FET than for fresh transfers in this period. The domain for IR is 2011–2014 because the CDC started reporting implantation rates for FET in 2011

63.1.3 A Brief History of Oocyte and Embryo Cryopreservation

The first scientific discussions of sperm cryopreservation date back at least to the nineteenth century, while the first low-temperature preservation of rabbit oocytes was published in 1948. Mouse embryos were successfully frozen in 1972.

The first human pregnancy with a frozen embryo was reported in 1983 [7]. That pregnancy was achieved in just 1 of 15 patients undergoing transfer of thawed cleavage-stage embryos. The embryo in that cycle was thawed 13 h before transfer to "allow recovery of cell function and a reassessment of cryoinjury" prior to transfer. Unfortunately, the ongoing pregnancy terminated at 24 weeks due to septic infection secondary to premature rupture of membranes.

The first live birth following FET was reported in 1984 [8]. Monozygotic dichorionic twins were born following the transfer of one intact thawed eight-cell embryo. The authors justified the development of FET methods, "The question of suboptimal uterine receptivity as a result of hormonal pretreatment has received little attention." Equally visionary, the first observation of greater success with frozen embryos than with fresh embryos in 1987 led to the conclusion that "…a normal pregnancy rate may result if the embryos originating from an unsuccessful IVF-ET cycle are transferred in a favourable uterus" [9].

Early cryopreservation protocols used conventional slow freezing of cleavage-stage embryos, with glycerol, dimethyl sulfoxide, propanediol, and/or sucrose frequently used as cryoprotectants to prevent intracellular ice crystal formation. By 2000, blastocyst cryopreservation was becoming routine due to much greater subsequent implantation rates than were realized with thawed cleavage-stage embryos [10].

Today, the predominant methods used are oocyte vitrification and blastocyst vitrification, while cryopreservation at other stages and conventional slow freezing are used less often. The ongoing rapid increases in success rates due to improving cryopreservation techniques have resulted in increasing popularity of the freeze-all cycle.

63.2 Indications for a Freeze-All Cycle

There are now numerous indications for the freeze-all cycle, and some of them will be described here.

63.2.1 Embryo-Endometrium Asynchrony

Because many human oocytes and embryos are nonviable, it is routine to collect many oocytes in order to increase the probability of obtaining one or more viable embryos through IVF. COS with exogenous gonadotropins is routinely used for this purpose. By developing numerous follicles, COS allows the collection of many mature oocytes and the subsequent creation of multiple embryos.

However, COS is associated with supraphysiologic serum levels of estradiol and with both early and supraphysiologic progesterone increase. These supraphysiologic elevations are found in nearly all COS cycles resulting in oocyte collection and result from the numerous large follicles that develop. Estradiol promotes endometrial development, and progesterone initiates endometrial maturation in preparation for the implantation of a hatched blastocyst, suggesting the potential for abnormal endometrial development under endocrine conditions altered by COS.

Studies have revealed that the human endometrium is histologically advanced after COS exposure, typically by about 2 days [11, 12]. This is significant because histologic maturity lasts no more than 2 days [11]. In other words, COS

typically displaces the period of histologic advancement and the putative implantation window by approximately the full duration of that window. Progesterone and its endometrial receptor appear to be in the causal pathway of this advancement. The degree of histological advancement correlates with pre-trigger progesterone levels [11, 13]. The endometrial progesterone receptor is downregulated about 2 days earlier in COS cycles [14]. Premature progesterone elevation correlates with advanced echogenicity [15] and implantation failure in fresh transfer [16, 17] but not if the embryos are cryopreserved and transferred in a subsequent cycle [18].

This evidence suggests that COS might create a bias toward implantation of rapidly developing embryos. This is precisely what has been reported clinically [19–24], and it seems no coincidence that embryo scoring algorithms developed over decades of fresh autologous transfer favor transfer of the most developed embryos. Furthermore, it has also been shown that slowly developing embryos, specifically day 6 blastocysts, have greater implantation rates after freezing and thawing than in fresh transfers [19, 21]. Because freezing and thawing are unlikely to transform nonviable embryos into viable embryos, the most plausible explanation seems to be that COS reduces endometrial receptivity for slowly developing embryos by advancing endometrial maturation and therefore inducing an asynchrony between the embryo and the endometrium.

Randomized trials have reported superior implantation and pregnancy rates in FET cycles when compared to fresh transfers following COS exposure [25–28], although in one trial, that difference was not significant [28]. A retrospective matched-pair comparison of fresh and FET cycles, matched on patient age, embryo morphology, and day of blastulation (day 5 or day 6), also found superior success rates with FET and that the benefit was much greater for (slowly developing) day 6 blastocysts than for their faster day 5 counterparts [24].

Performing a fresh transfer in cases of embryo-endometrium asynchrony following uterine COS exposure risks wasting the best embryos, perhaps the patient's only embryos, in a non-receptive uterine environment. Therefore, it is often better to freeze the entire embryo cohort for later use than to transfer fresh embryos. However, embryo cryopreservation risks loss of embryos through cryodamage. The choice of whether to transfer fresh or to freeze all embryos or oocytes must balance these two effects while considering the efficacy of the laboratory methods and personnel. Current vitrification techniques are associated with very low risk of embryo loss.

Multiple studies have shown that premature progesterone elevation corresponds with increased risk of implantation failure [16, 17, 22, 29]. Premature progesterone elevation further advances endometrial development and further exacerbates the embryo-endometrium asynchrony induced by COS exposure. However, premature progesterone elevation can also correspond with larger follicles and superior oocyte and embryo cohorts, partly mitigating the asynchrony effect. The solution is to cryopreserve embryos in cycles with premature progesterone elevation. This solution has been shown to significantly increase implantation and pregnancy rates [18].

Slow embryo development, such as with blastocysts that fully expand on day 6 instead of day 5, has an effect that is similar to premature progesterone elevation [22]. The two effects together are substantially worse than either effect alone [22, 29]. Embryo cryopreservation followed by FET has been shown to increase implantation and pregnancy rates of slow-growing embryos [24]. Because cryopreservation is not believed to improve the embryos themselves, the putative cause of this effect is superior synchrony between the embryo and endometrium in FET cycles.

Some may believe that the issues discerned by the comparisons of day 5 and day 6 blastocysts are not relevant in cleavage-stage embryo transfer. Transfer of cleavage-stage embryos cannot negate the effects of delayed blastulation. While transfer on day 3 blinds the clinician to the day of blastulation, the embryos still must blastulate and hatch before implantation is possible. Day 3 transfer cannot restore synchrony for slowly developing embryos and therefore cannot change their fates. Pregnancy and birth rates with cleavage-stage transfer can be comparable to those of day 5 blastocyst transfer only if extra cleavage-stage embryos are transferred to compensate for their unknown blastulation potential and unclear synchrony. The transfer of extra embryos increases the risk of multiple pregnancy, simultaneously increasing numerous maternal and perinatal risks. As synchronous single-blastocyst transfer becomes the norm, the transfer of multiple cleavage-stage embryos to compensate for their lower implantation potential grows obsolete.

63.2.2 Normal Responders

One randomized trial has compared fresh transfer and FET success rates in normal responders [25], defined as having 8–15 antral follicles observed on ultrasound. Patients were also required to be 18–40 years of age, to have cycle day 3 FSH <10.0 IU/L, and to be undergoing their first IVF cycle. That study employed conventional slow freezing of entire cohorts of bipronuclear oocytes, followed by thaw of entire cohorts and extended culture to the blastocyst stage. Patients in both study arms had two blastocysts transferred when available, and genetic testing of embryos was not allowed. These methods were chosen so that each study arm would have best-of-cohort blastocysts transferred and so that post-thaw extended culture could eliminate from consideration any embryos that were too damaged by slow freezing to resume development.

In that study of normal responders, the FET arm had significantly greater success rates than did the fresh transfer arm. These measures included the implantation rate (70.8% vs. 38.9%), the clinical pregnancy rate per transfer (84.0% vs. 54.7%), and the ongoing pregnancy rate per transfer at 10-week gestation (78.0% vs. 50.9%). Because the main outcome measure (clinical pregnancy) met the stopping criterion at this interim test point, the study was halted early, after 103 patients were completed. The researchers concluded that

the results "strongly suggested" impaired endometrial receptivity following COS exposure.

The high implantation rates in that study indicate the FET method is ideal for single-blastocyst transfers.

63.2.3 PCO Patients and High Responders

Another randomized trial compared fresh transfer to FET in a population of patients 20–34 years of age meeting the Rotterdam criteria for polycystic ovary (PCO) syndrome [26]. This study employed cleavage-stage embryo transfer and cleavage-stage vitrification.

The 1508 randomized patients included 746 randomized to freeze-all and 762 randomized to fresh transfer. Of those, 49.3% and 42.0% achieved live birth, respectively (RR 1.17, 95% CI 1.05–1.31). The authors concluded that PCO patients undergoing freeze-all have a greater chance of live birth than those undergoing fresh transfer. They also noted reduced OHSS risk in FET, but also greater risk of preeclampsia.

Another randomized trial compared fresh transfer and FET in high responders [28]. This study found clinical pregnancy rates per transfer of 79.6% with FET and 65.4% with fresh transfer. The RR for clinical pregnancy was therefore 1.22, which was similar to that of the larger study in PCO patients [26]. However, in this smaller study, those observed differences were not statistically significant.

63.2.4 Prior Implantation Failure with Fresh Embryos

In 1987, Testart retrospectively studied 63 fresh transfers and 77 FETs and reported lower pregnancy rates following fresh transfers of primary embryos (10.5%) than in transfers of frozen-thawed supernumerary embryos (27.1%) following failed fresh transfers and concluded that "uterine inadequacy" in some patients following COS could be addressed through FET into a "favorable uterus" [9].

Another retrospective study compared fresh cycles with freeze-all cycles in a population of patients with prior implantation failure following at least one fresh blastocyst transfer. In this study, 163 patients opted for another fresh cycle, while 106 opted for freeze-all followed by FET. Patients opting for freeze-all followed by attempted FET had 46.2% live birth rate per retrieval, while those opting for another fresh cycle had only 21.5% live birth rate per retrieval. The authors concluded that patients with prior implantation failure with fresh embryo transfer had a significantly greater chance of live birth with freeze-all than with another fresh cycle [30].

63.2.5 Advanced Age

A retrospective analysis examined fresh autologous transfer and found the risk of having at least one indicator of embryo-endometrial asynchrony, including elevated preovulatory progesterone and/or delayed (day 6) blastulation, increased with increasing patient age [31]. Specifically, this risk increased from 50.0% in patients <35 years of age to 68.1% in patients ≥35 years old.

Live birth rates decreased when asynchrony was indicated, such that 62.9% of synchronous transfers in patients <35 resulted in live birth, but only 27.9% of patients had live birth when day 6 blastocysts were transferred after elevated progesterone was observed. In patients ≥35, those rates were 38.9% and 18.1%, respectively.

The age-related negative effects of COS on success rates following fresh transfer may explain why the national average success rates in FET (Figs. 63.2 and 63.4) have been consistently less disperse across age groups than those in fresh transfer (Figs. 63.1 and 63.3).

63.2.6 Risk of Ovarian Hyperstimulation Syndrome

Ovarian hyperstimulation syndrome (OHSS) can occur when numerous corpora lutea, such as seen in high responders or PCO patients, have prolonged stimulation by human chorionic gonadotropin (hCG). hCG from an ovulatory trigger injection can induce early-onset OHSS, while hCG produced by implanting embryos in early pregnancy can result in late-onset OHSS.

There are several published methods to reduce the risk of early-onset OHSS. The most effective of these, other than outright cycle cancellation, is to trigger with a GnRH agonist instead of hCG [5]. The agonist induces an immediate surge of pituitary LH, which matures the oocytes much as hCG would. However, LH has a much shorter half-life than hCG. The rapid decline in LH levels typically results in rapid and irreversible luteolysis, such that any developing signs of OHSS following agonist trigger rapidly fade shortly after oocyte collection, in marked contrast with the sustained and worsening OHSS symptoms that can follow hCG trigger.

There are limitations to the use of agonist trigger. First, the agonist is only able to induce an LH surge if daily agonist is not used for pituitary downregulation. Second, OHSS remains possible, although at a greatly reduced rate when compared to hCG trigger. Lastly, the success rates with fresh transfer are reportedly compromised following agonist trigger [32]. The reduced success rates following fresh transfer make the use of agonist trigger an indication for freeze-all cycles [33, 34].

Reducing the risk of late-onset OHSS, especially after an hCG trigger in a high responder, generally requires freezing all embryos for later use in another cycle.

A randomized trial in PCO patients found the overall risk of OHSS was reduced (1.3% vs. 7.1%, respectively; RR = 0.19, 95% CI 0.10–0.37) if all embryos were frozen in lieu of fresh transfer following ovulatory hCG trigger [26].

Elevated OHSS risk is therefore an indication for freeze-all, regardless of whether hCG or GnRH agonist is used for the ovulatory trigger.

63.2.7 Preimplantation Genetic Screening

PGS is increasingly used to select the most viable embryos for transfer. With PGS, a biopsy of one or more cells is taken from each embryo and is then analyzed for chromosome copy number. Embryos with the normal number of chromosomes are eligible for transfer. Success rates with trophectoderm biopsy of blastocysts are reportedly improved over transfer of untested embryos [35].

However, the genetic testing is typically performed by a specialized laboratory distinct from the clinic itself. The embryos must be shipped to that laboratory, and the results become available at least 1 day later. Holding an expanded blastocyst in growth media for 1 or more extra days, to the fully hatched stage and beyond, might exceed the embryo's implantation window. It is therefore common to freeze all embryos in cycles employing PGS.

A randomized trial compared fresh and freeze-all cycles in 179 patients using next-generation sequencing for PGS. The fresh group had day 5 blastocysts biopsied, cultured overnight, and transferred on the morning of day 6. The freeze-all group had day 5 and day 6 blastocysts biopsied before vitrification. The fresh group had significantly lower live birth rates than did the freeze-all group, on a per transfer basis (59% vs. 77%) or per intent to treat (40% vs. 62%) [27]. Similar conclusions were already reached with retrospective analysis [36] and literature review [37].

63.2.8 Oocyte Banking

Cryopreservation of oocyte cohorts is increasingly common. One purpose is for women who wish to preserve their fertility, such as before oncotherapies or as they approach advanced age. Chemotherapy and radiotherapy are established threats to ovarian function. Cryopreserving oocytes is one option for preserving the chance of pregnancy with autologous oocytes.

Another purpose of oocyte cryopreservation is for donor egg banks. Donor egg banks provide a less costly means for patients to obtain donor eggs when compared to traditional fresh oocyte donation cycles in which the donor and recipient have synchronized cycles.

63.3 Risks in Fresh Transfer and FET

63.3.1 Maternal Risks

Fresh embryo transfer is associated with altered maternal risks when compared to FET.

A randomized trial showed that fresh transfer following hCG trigger in PCO patients increased OHSS risk when compared to freeze-all [26]. This makes physiologic sense, as a fresh transfer allows hCG produced by implanting embryos to stimulate the corpora lutea, which can induce late-onset OHSS or sustain early-onset OHSS. Freezing all embryos for use in a later cycle precludes those possibilities, as the follicles will luteolyze after clearance of the hCG trigger.

Fresh transfer following COS exposure is also associated with increased risk of ectopic pregnancy when compared to FET [38–40]. One physiologic explanation for this is that the elevated levels of estradiol and progesterone associated with COS alter uterine contractility and tubal ciliary motions, facilitating embryo displacement. Another explanation is that impaired endometrial receptivity following COS exposure leaves unimplanted embryos available to potentially implant elsewhere, such as the tubes. These mechanisms could be cooperant.

Fresh transfer may alter other maternal risks. Increased risks of placenta previa, placental abruption, and antepartum hemorrhage were associated with fresh transfer in meta-analyses [41], while one randomized trial reported reduced risk of preeclampsia with fresh transfer [26]. However, FET has been associated with increased use of caesarian delivery.

Overall, the current literature suggests FET has reduced maternal risks when compared to fresh autologous transfer.

63.3.2 Perinatal Risks

Fresh autologous embryo transfer following COS exposure is also associated with altered risks to the fetus and offspring, when compared to FET.

Increased risks of preterm birth, low birthweight, being small for gestational age, and perinatal mortality have been associated with fresh transfer in meta-analyses [41]. However, reduced risk of macrosomia has also been associated with fresh transfer [42].

Randomized trials have reported 162–166 g lower mean birthweight with fresh transfer when compared to FET [26, 43]. This birthweight difference probably explains the greater risk of low birthweight, the reduced risk of macrosomia, and the reduced use of caesarian deliveries that have been associated with fresh transfer when compared to FET. A large retrospective study of singleton birthweights in Australia [44] found singleton birthweights were reduced by 156–167 g in fresh transfer when compared to natural conception or FET. Natural conception and FET did not differ significantly in singleton birthweight.

Other large retrospective comparisons of singleton birthweights found that birthweights were similar following fresh and frozen-thawed embryo transfers in oocyte donation cycles in which neither group had uterine exposure to COS [45, 46], contradicting an embryonic effect of cryopreservation on birthweight.

Therefore, the existing evidence points to a uterine effect of COS exposure on birthweight. This is further supported by the association between COS and abnormal placentation seen in a rodent model [47].

It has long been known that low birthweight is more prevalent among singleton births following IVF than in the general population of singleton births [48]. It is also known

that birthweights in the general population are already less than the birthweight corresponding with minimal infant mortality [49]. This suboptimal state of the general population might be because a high proportion (37–51%) of births in the general population follow unintended pregnancies and because the incidence of low birthweight is increased by unintended pregnancy [50–52].

All IVF pregnancies are intended; therefore, the ideal reference group includes only births following intended pregnancies, where the incidence of low birthweight is reduced. The use of a reference group with greater birthweights would clarify that birthweights following fresh transfer are reduced and low birthweight incidence is increased to even greater degrees than previously estimated. Therefore, it seems likely that the ~165 g greater mean birthweight following FET is an improvement over that of fresh transfer, despite the increased risk of macrosomia and the increased use of caesarian delivery with FET. However, this hypothesis awaits proper study to compare the frequency, cost, and health effects of relevant risks.

Increased epigenetic DNA methylation has been associated with fresh autologous transfer, but not FET, when compared to natural conceptions [53].

63.4 The Changing Paradigm of Assisted Reproduction Through the Freeze-All Strategy

63.4.1 The Ongoing Paradigm Shift

The proportion of freeze-all (banking) cycles reported by the Centers for Disease Control and Prevention has recently increased (Fig. 63.6), as has the number of transfers that use frozen embryos. The number of fresh transfers has decreased.

No medical organization has openly endorsed or encouraged this paradigm shift. The shift can only be attributed to clinicians and patients mutually recognizing the advantages of freeze-all and FET in the medical literature and/or at their own centers as cryopreservation techniques continue to improve.

63.4.2 Optimizing the Freeze-All Cycle

To date, COS protocols for freeze-all cycles have been largely the same, those developed and routinely used for fresh transfer cycles. Every randomized trial comparing fresh and freeze-all cycles has used the same COS protocol for the fresh transfer arm as for the freeze-all arm. These protocols evolved over the decades of fresh autologous transfers and are therefore optimized for fresh transfer cycles. Such protocols are designed to obtain a useful cohort of viable oocytes and embryos for fresh transfer without impairing endometrial receptivity. These are conflicting goals, given the advanced endometrial receptive phase associated with COS, and therefore both goals are typically compromised.

The traditional COS protocol includes an ovulatory trigger once two to three lead follicles reach 17–18 mm, leaving many developing follicles somewhere <17 mm in size at the time of trigger. However, it has been shown that larger follicles, those larger than 18 mm, are more likely to yield mature oocytes, fertilized oocytes, and day 3 embryos with the least fragmentation and that oocyte maturity and fertilization potential increased steadily with greater follicle size [54]. This suggests a trigger based on lead follicle size of 17 or 18 mm compromises the oocyte cohort.

It has further been shown that extending stimulation so that more oocytes are collected does not increase success rates in fresh transfers, putatively because preovulatory

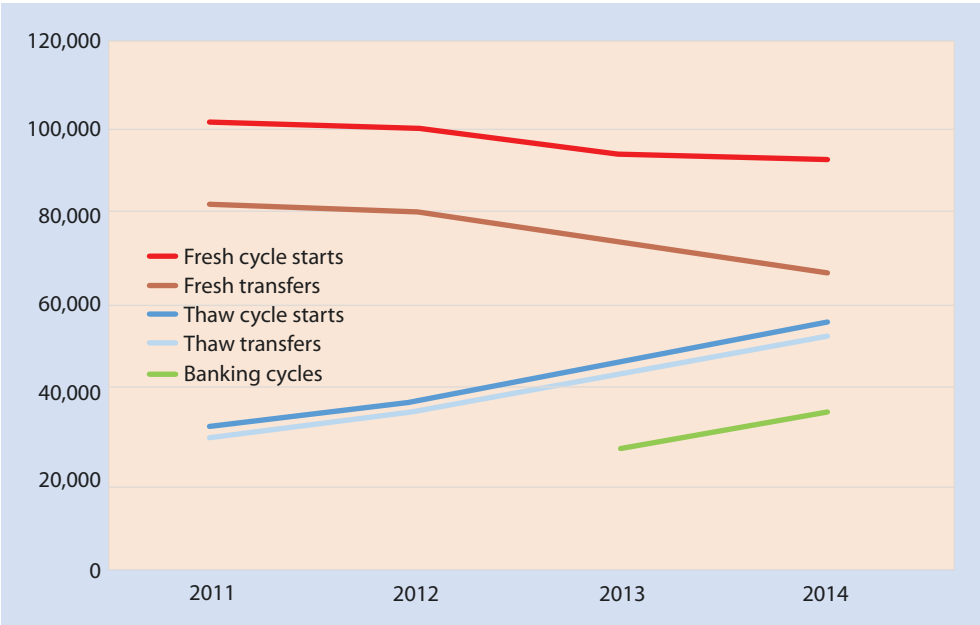

Fig. 63.6 Trends in national numbers of reported autologous cycles from 2011 thru 2014. Note the decreasing numbers of reported fresh cycle starts and fresh transfers and simultaneously increasing numbers of thaw cycles, thaw transfers (FETs), and banking cycles. The domain for the trend in banking cycles is limited to 2013–2014 because the CDC started reporting this statistic in 2013 and 2014 is the latest data available as this is written

progesterone levels increase and impede endometrial receptivity [55]. This is not an issue in freeze-all cycles, and the COS protocol can be adjusted to optimize the oocyte cohort without regard to endometrial receptivity. This has yet to be tried in any randomized trial, however.

Furthermore, the cost of COS can be reduced in freeze-all cycles. For example, GnRH agonists and antagonists are routinely used to prevent premature LH surges. However, much cheaper oral progestogens have been shown to work as well at preventing LH surges, presumably by controlling hypothalamic GnRH secretion [56]. Such use of progestogens is incompatible with fresh autologous transfer because endometrial receptivity would be impaired. However, for freeze-all cycles, the lack of endometrial receptivity is irrelevant. Therefore, the COS protocol for freeze-all cycles can take advantage of the reduced cost and simpler administration of oral progestogens when compared to GnRH agonists and antagonists. The use of progestogens to control the hypothalamus retains the option of using GnRH agonist as the ovulatory trigger in patients at risk of OHSS.

However, a COS protocol optimized for the freeze-all strategy might be suboptimal for fresh transfer cycles and might even preclude implantation following fresh transfer. Therefore, the use of such optimized protocols may require routine freeze-all cycles.

63.4.3 Impact of Freeze-All Strategies on Laboratory Operations

IVF laboratory techniques have changed dramatically in the last four decades. These changes include more controlled and complex embryo culture media providing the basis for in vitro blastocyst culture. PGS, when used as an embryo selection criterion, and improved cryopreservation technique by vitrification further allow increased pregnancy rates per transfer and per cohort.

These techniques require skilled professionals, training, detailed operating procedures, appropriate laboratory facilities and equipment, controlled laboratory conditions, and quality control.

Embryo cryopreservation techniques themselves have evolved. In the 1990s, pre-embryos were frozen in media containing DMSO as cryoprotectant using the slow freeze and slow thaw method using the controlled-rate freezing unit. In those cases, thawing a single vial for a frozen embryo transfer could take hours. Initial results were encouraging, but pregnancy rates were still low, especially by modern standards. Gradually, the use of propanediol and glycerol as cryoprotectants combined with non-permeating sugars like sucrose became standard [10]. For many years, conventional slow freezing techniques provided reasonable survival and success rates. With controlled-rate freezers, many embryos from different patients could be frozen simultaneously during a period of 3–4 h. Different freezing curves and cryoprotectants were tried for various embryo stages. The freezing curves basically avoided the formation of intracellular ice, while the embryos were subject to a dehydration process. The embryos were thawed by a rapid thaw in air, followed by a water bath, and then taken through different solutions of decreasing concentrations of the cryoprotectant. By about 1995, embryo thaw and transfer became an easy task for well-trained personnel.

The embryology workload increased with PGS combined with vitrification in the modern laboratory. Modern trophectoderm biopsy requires the highest level of training and typically also requires blastocyst vitrification.

Vitrification now yields survival rates near 100% and very high pregnancy rates. Several commercial vitrification solutions are commercially available.

The technique of cryopreservation has an interesting relationship with laboratory workload. One embryo can be vitrified in <10 min. Freezing one or a few embryos from one cohort is typically much faster with vitrification than with conventional slow freezing. However, vitrification requires constant attention from the embryologist(s), while a controlled-rate freezer does not. Conventional slow freezing becomes easier than vitrification when large numbers of embryos are frozen from multiple patients each day, such as with routine cryopreservation of all cohorts at the bipronuclear stage at a center with many oocyte retrievals daily.

A busy freeze-all program can require multiple dedicated embryologists. Also, vitrification devices are at least ten times as expensive as those for slow freezing, and the commercial vitrification solutions are also relatively costly. Freezing one embryo per device, as required after blastocyst biopsy and in centers where eSET is the standard of care, increases the number of devices required and the space required per patient in the liquid nitrogen storage tanks.

In addition, the freeze-all program also impacts laboratory dynamics. Embryo thaws may need to be performed early in the morning if hours of observed re-expansion are required to confirm survival. The success of a modern freeze-all laboratory is measured by rates of blastocyst formation, post-thaw survival, thawed blastocyst utilization, implantation (fetal hearts per transferred embryo), sustained implantation (live birth per transferred embryo), and perinatal outcomes. Traditional live birth rates and pregnancy rates are less useful if multiple embryos are transferred, as the success rate can be manipulated by transferring multiple embryos to compensate for poor technique.

63.4.4 Freeze-All and PGS as Synergistic Pathways to Elective Single-Embryo Transfer in All Patients

Successful IVF requires the transfer of a viable embryo into a synchronously receptive uterine environment. The greater implantation rates with FET in randomized trials suggest embryo-endometrium synchrony is improved in artificially prepared cycles when compared to COS cycles.

Trophectoderm biopsy and PGS support the selection of embryos that are most likely to implant [35]. However, PGS results typically require a day or more to arrive, and this delay often compels the freezing of all embryos. PGS, freeze-all, and FET are therefore often used together.

Increasing implantation rates make eSET practical and more acceptable and desirable to patients. As implantation rates increase beyond 50%, the pregnancy rate is less affected by the number of embryos transferred. Theoretically, at 100% implantation rate, all transfers would result in pregnancy regardless of the number of embryos transferred, removing any motivation for multiple-embryo transfer for patients desiring one child at a time.

Multiple-embryo transfer greatly increases the risk of multiple pregnancy. The increased risks associated with multiple pregnancy are well known and include maternal death, premature birth, low birthweight, perinatal death, blindness, deafness, and cerebral palsy, among others. The financial costs to society and parents are significant. If the multiple pregnancies resulting from IVF had been singletons, the estimated cost savings for insurance claims alone would have been approximately four times the total cost of IVF services in 2004–2009 [57]. There are additional costs not covered in that analysis, such as increased child-care costs or when multiple birth compels the purchase of a larger vehicle, a larger home, or a parent to forego a career in order to care for multiple infants.

Therefore, any reasonable and safe method that improves the implantation rate and therefore encourages eSET should be applied and should be endorsed by medical organizations.

63.4.5 Cost-Effectiveness of the Freeze-All Strategy

At first, it may seem the freeze-all strategy would be more expensive than a fresh transfer strategy, given the added costs associated with cryopreservation. However, the cost per cycle is not the most relevant statistic, as patients seek fertility treatment to have children, not cycles. The cost per pregnancy or per live birth would be much more meaningful statistics for comparing cost-effectiveness.

To date, there have been two published comparisons of the cost-effectiveness of the freeze-all strategy and the fresh transfer strategy. Both studies found the freeze-all strategy to be more cost-effective than the fresh transfer strategy.

One study of 530 cycles reported the cost per ongoing pregnancy was about 18% greater with a fresh transfer strategy when compared to freeze-all [58]. The other study of 252 cycles reported the cost per live birth was 17% greater with a fresh transfer strategy than with freeze-all [59]. Further research should also consider costs associated with maternal risks (e.g., OHSS, ectopic pregnancy, preeclampsia) and perinatal risks (e.g., prematurity, low birthweight, macrosomia). Current evidence suggests most of these risks are more prevalent with fresh transfer, and therefore their consideration might make the fresh transfer strategy appear even less cost-effective.

Further cost benefits should be realized as the increasingly greater implantation rates of FET encourage increased use of elective single-embryo transfer (eSET), reducing the elevated risk of multiple pregnancy that has long been associated with IVF. The implantation rate with transfer of vitrified-warmed blastocysts is reportedly as high as 70% in a group of patients 18–40 years of age with at least normal ovarian reserve, even without PGS [60]. This rate is ample to support eSET.

63.4.6 New Opportunities

The freeze-all strategy creates new opportunities and treatment paradigms.

One example is the opportunity to bank multiple cohorts of oocytes or embryos before attempting transfer. This might be advantageous, for example, in an older patient who produces few oocytes and is opting for PGS or PGD. In older patients, a single oocyte cohort has only a modest chance of yielding a morphologically and genetically acceptable blastocyst. By banking multiple cohorts, the derived blastocysts can be tested in one group in order to take advantage of package pricing for PGS while also increasing the chance of having at least one euploid blastocyst to transfer. This option is impossible under the classic fresh transfer paradigm.

A similar example arises for women desiring multiple children. A woman of advanced age is unlikely to produce multiple viable blastocysts from one retrieval. If she achieved pregnancy and live birth in the fresh transfer paradigm, it would typically be years before she returned to try again. Her ovarian reserve could decrease significantly in that interval, making subsequent births problematic. Alternatively, under a freeze-all paradigm, she could repeatedly bank embryos until the number and quality of stored embryos indicated a high probability of achieving her goals and then proceed with a series of thaws, single-embryo transfers, and births until her goals are achieved.

63.4.7 Summary and Needed Research

Current evidence indicates generally improved success rates, reduced costs, and reduced maternal and perinatal risks with the freeze-all strategy when compared to fresh transfer.

Randomized trials comparing success rates are still needed in some populations, including low responders. Future research should consider cumulative live birth rates per oocyte retrieval.

Further research should investigate the greater flexibility of COS protocols when no fresh transfer is planned, as originally discussed by Edwards and Steptoe in 1977 [3]. This allows oocyte and embryo cohort optimization unrestricted

by endometrial receptivity concerns. Maternal and perinatal risks must be minimized, such as through GnRH agonist trigger [33, 34]. Patient costs must also be minimized. Societal costs of the entire process should be minimized, such as through routine elective single-embryo transfer [57]. New research of the cost-effectiveness of the freeze-all strategy should consider costs associated with maternal and perinatal risks.

Future investigation of birthweight effects should transition from comparing birthweight distributions following fresh transfer, FET, or the general population of all pregnancies to instead comparing ideal birthweight distributions based on minimized overall risk [49]. If a control group of natural conceptions is to be used, it should be restricted to intended pregnancies [50–52].

Review Questions

1. To date, randomized trials have compared success rates following fresh transfer and freeze-all strategies in all of the following populations except:
   A. Low responders
   B. Normal responders
   C. High responders
   D. PCO patients
   E. Patients opting for PGS

2. Based on current evidence, which of the following risks is/are reduced by the freeze-all strategy and FET when compared to fresh transfer?
   A. OHSS
   B. Ectopic pregnancy
   C. Low birthweight
   D. Perinatal mortality
   E. All of the above

3. Based on current evidence, which of the following risks is/are increased in singleton pregnancies following FET when compared to fresh transfer?
   A. Placenta previa
   B. Placental abruption
   C. Macrosomia
   D. Prematurity
   E. All of the above

Answers to Review Questions

1. A

2. E

3. C

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Management and Regulation in the ART Laboratory

Contents

IVF Data Management: From Clipboards to Smart Apps

Jacques Cohen, Stephen Fiser, and Giles Tomkin

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Learning Objectives

- To evaluate different facets of data management in clinical medicine and assisted reproduction in particular
- To examine shortcomings of current electronic medical recordkeeping systems for clinical medicine and assisted reproduction and approaches how to address them
- To discuss the sparsity of cloud-based applications in assisted reproduction
- To present the first QC app for laboratories and compare its structure to generic utility apps

64.1 Introduction

There is a tremendous amount of data handling and paperwork in clinical medicine [1]. When only paper is used, the task of organizing, controlling, and safekeeping patient records is demanding and subject to varying degrees of error. Transcription inaccuracies are very common, even when prescribing drugs [2]. A significant body of evidence points to such medical paperwork errors as a leading cause of iatrogenic death and injury [3, 4]. The inaccuracy of information technology is also blamed for other adverse clinical events. This is largely due to the absence of "interoperability," rather than a shortage of time spent by professionals on collection and recording of information. The Healthcare Information and Management Systems Society (HiMSS) defines interoperability in healthcare as "the ability of different information technology systems and software applications to communicate, exchange data, and use the information that has been exchanged" (► http://www.himss.org/library/interoperability-standards/what-is?).

The outdated and inefficient paper-based bureaucracy simply does not allow interconnectivity or interoperability.

It is estimated that doctors and other healthcare professionals spend close to 50% of their time shuffling through paper charts, forms, messages, and reports [5]. Improved scrutiny and oversight through nongovernmental and governmental inspections and audits are clearly increasing this burden. This is especially true in assisted reproduction where laboratory staff may spend even more time on paperwork than other team members. It is estimated that only 50% of IVF clinics have implemented an electronic medical record (EMR) system, although this figure is difficult to confirm since no studies have been published on the topic.

64.2 Complexity of ART and Streams of Paperwork

Assisted reproduction is the only medical intervention that is designed to treat not just individuals but couples or multiple participants, including, for instance, gamete donors and gestational carriers. To make matters even more complicated, these multiple individuals have associated with their gametes and embryos, the development of which must be monitored and recorded meticulously during several days in the laboratory. The monitoring of procedures, observations, and dispositions generates staggering amounts of information and paperwork for each patient, embryo, and procedure. Yet, order can be established in what may look to the unfamiliar like an impossible system integrating patients, gametes, embryos, lab procedures, assays, surgery, counseling, and more. Patients often undergo multiple attempts, but what may be considered standard treatment in one clinic or country can be considered illegal or unconventional in another locale. Generally speaking, there are three streams of data, more or less integrated, each with a potential software solution: (1) the EMR, which is a system for tracking patient information and providing clinical care; (2) an "enterprise software" system which is a set of tools that satisfies the needs of an organization like a hospital, laboratory, or clinic; and (3) the quality management (QM) system which generates data sets requiring customized forms and special standard operating procedures. Whereas electronic data management systems exist for the first two sources of data, QM remains largely confined to forms, paperwork, and clipboards.

64.3 The EMR System

64.3.1 General Characteristics

The first data stream involves patient procedures and attempts to achieve pregnancy. This includes monitoring follicular development in the female partner, the administration of drugs, and the surgical procedures. Later, individual gametes and embryos are tracked, although in many labs, embryos are grown in groups so that individual identity within the cohort is lost. This flow of information can be captured with a tailored EMR, often referred to as an electronic health record (EHR). An EMR is an ordered collection of patient health information data in a digital format. It becomes increasingly complex when records are shared across different healthcare settings. Sometimes, this can take the form of an entire country's healthcare records (e.g., national registries exist in Sweden, France, Belgium, the Netherlands, and other countries). Records are shared through network-connected information systems. EMRs may include a range of patient parameter data, including personal data and demographics, medical history, medication and allergies, vaccination status, laboratory test results, radiology images, vital signs, and billing information. EMRs for assisted reproduction have the added complexity of tracking two or more individuals throughout their treatment and following their eggs, sperm, and resulting embryos individually or in groups. The beginning of procedure and data input activity is usually straightforward, but because gametes and embryos can be cryopreserved and later thawed across multiple attempts, which may be combined for various reasons, the end of treatment is often unclear and assignment of final embryo dispositions can be delayed. Also, gametes and embryos, donated or shared, can be imported or exported from and to other clinics within or between countries.

At a minimum, an ART EMR should be able to monitor key performance indicators (KPIs). For instance, clinic management teams should be able to assess periodic fluctuations in egg maturity, fertilization rates, embryo development, blastocyst formation, implantation, miscarriage, and live birth rates. Preferably, the data should be sortable by attempt, age, staff, etiology, and procedure type. Existing EMR systems generally do not have automatic error detection capabilities and cannot flag incorrect or missing data; some systems even lack any detailed reporting features. These deficiencies are especially perplexing because subscription fees to assisted reproduction EMR systems are quite high, naturally increasing total treatment cost to the patient. Laboratory instrumentation tracking and possible correlation with outcomes are absent in most EMRs. ART, oddly enough, has not yet embraced improvements in data handling through cloud-based applications (apps) but more on that later.

64.3.2 Safety and Privacy

Safety of data storage and privacy of data are clearly paramount. However, some clinics are still using single copies of paper medical charts in unsecured cabinets. Some EMRs have no privacy settings such as those defined by the Health Insurance Portability and Accountability Act (HIPAA) of 1996 in the USA and a law of the same name in the UK since 1998 under the Data Protection Act of 1998. Similar laws exist in Canada, the Personal Information Protection and Electronic Documents Act (PIPEDA or PIPA), and Australia with the early Privacy Act of 1988 amended in 2012 to cover Internet-based data handling.

64.3.3 EMR Objectives and Limitations

EMR systems should be designed to collect and store all relevant data and to capture the status of a patient's ART attempts across time. They should obviate the need to track down and search a patient's paper records for information, particularly if the data are properly validated and accuracy is established, perhaps through multilevel filtering and interactive reporting. EMRs should reduce data duplication, keep the patient file up to date, and decrease the risk of information loss. A major advantage of EMRs is that digital data, even when encrypted, are searchable and analyzable and may be copied at any time. This is achieved by reporting and filtering features, which should always be integral parts of the EMR. However, unfortunately, this ideal scenario is often not actual, and users may complain about either the lack of reporting features or their impracticality. Due to digital sorting and searching, EMRs can be very effective when extracting medical data for the examination of possible trends and fluctuations in outcomes over time. An EMR without search features and clever reporting is narrow and not much more useful than a written record system. EMR user satisfaction levels are mostly anecdotal; no systematic survey had been done until 2013 or published by individual EMR providers, but general attitudes and complaints regarding the use of EMRs have at last been detailed and studied after the American Medical Association (AMA) commissioned the Rand Corporation with a large investigation, the results of which were published in 2013 [6]. The study focused particularly on physician and nurse satisfaction with EMRs. More than 100 physicians in different disciplines were interviewed and surveyed extensively. The study demonstrated that most health experts were aware of the potential benefits of electronic tracking of patients such as remote medical record access; however, at least ten major complaints were identified in the Rand study. The most common areas of concern involved the differences in practice and clinical care between clinics and physicians. EMRs are often delivered "as is" meaning that end user-specific needs – for instance, having to do with workflow – can only be accommodated by clinic-specific customization, a process which is both time-consuming and expensive as it requires new coding or recoding. Other complaints concerned the complexity of systems and the fact that this complexity interfered with face-to-face care and that there was insufficient external health information exchange and information overload such as frequent alerts. Importantly, physicians felt that the systems did not improve over time. The more complex the EMR, the greater the dissatisfaction expressed. The situation described in the Rand study is reminiscent of that in assisted reproduction. Complaints about EMRs used in IVF are plentiful, yet the subject has not been "formally" studied. Some IVF groups still avoid using an EMR or switch between EMR providers hoping that this would solve their problems. ART EMRs are often based on outdated computer languages, data input, and local network systems. Dynamic reporting appears unattainable. To address these issues, flexibility or allowance for "dynamic input" is key when designing new generations of EMRs for ART.

64.4 Enterprise Software

The second stream of IVF information includes organizational and administrative data, such as that of any large organization or enterprise. Enterprise software satisfies the needs of the organization rather than the individual users. Such organizations include businesses, manufacturing plants, schools, charities, governments, universities, and clinics. Services provided by enterprise software are typically business-oriented tools such as online purchasing and payment processing, automated billing, security, IT management, client services, project, collaboration and human resource management, manufacturing, occupational health and safety, application integration, and forms automation. Many of these aspects are needed in the IVF environment; however, rarely are any such enterprise software systems developed for independent IVF clinics. A possible drawback is the cost and the need to train and test staff. Enterprise software is often available as a suite of customizable programs. The complexity of these tools, indeed the system as a whole,

demands expertise and specialized knowledge, which often come at prohibitive costs. A potential solution in IVF is the incorporation of some practice management tools into the EMR.

64.5 Laboratory and Program Quality Management

The third information stream in ART is the quality management (QM) program for andrology, embryology, and chemistry laboratories. Organized and electronic recordkeeping is mostly lacking in this area. Quality control (QC) of laboratory instruments and activities most commonly involves multiple forms which are completed on a daily basis by hand, or, less often, directly into a "makeshift" Excel sheet and very rarely compiled in a way that allows effective and timely analysis and reporting. QC data review typically occurs on a periodic basis, and the paperwork is only partially tracked and audited during annual laboratory inspections. Rarely do labs scrutinize the data to gain insight into instrument performance, unless some catastrophic event occurs. This is partly because the format of data collection makes such an effort unreasonably time-consuming, mostly uninformative, and difficult. Fluctuations in instrument performance may therefore be overlooked. Filing handwritten forms in cabinets or binders (usually for a finite minimum period as defined by law) is an outdated system, which basically means that large amounts of important data are being discarded.

64.6 New Opportunities: Big Data and Cloud-Based Applications

According to IBM, the world population is now creating 2.5 quintillion bytes of data on a daily basis. That means that 90% of the world's data has been created within the past 2 years [7]. This has placed an incredible demand on technologies designed to store and analyze data. On the bright side, the new technologies in combination with the explosion of mobile devices have created a unique opportunity to improve the state of affairs in the laboratory. Instead of transcribing information on forms attached to a clipboard and entering the data into an outdated system at a later point in time (or usually not), laboratory staff should be able to enter or dictate the data on a phone or tablet while they work – mostly eliminating errors that occur due to a messy multistep process – and generate a searchable, permanent, and immediately useful electronic record.

Rightly or wrongly, there are no international uniform requirements for the setup and maintenance of laboratories and equipment. This means that any existing software such as Excel or statistical packages will be suitable for some situations, but not for others, which in turn means the reports generated by these varied programs will be too different for comparison. Thus, each clinic will follow its own standards.

64.7 An Example of a QC App

We have recently released an app (Reflections™ by IVFqc) that allows customization of quality control for any laboratory instrument and associated parameters. The app is accessible from all Internet-connected devices (computer, tablet, or smartphone of any make) and not only does away with the clipboard but also allows for real-time fluctuation tracking and detailed reporting. The app can be applied to QC programs in any type of laboratory and is malleable to almost all laboratory systems. It has a so-called generic JSON API, which means that it allows connection to other software and hardware with relative ease. Some early adopters of the Reflections™ app are laboratory directors who run multiple laboratories and can now conveniently and efficiently track each of their laboratory's QC activities from their phone, switching from lab to lab with just a few clicks. The standard of security is based on HIPAA, as well as two-factor authentication and encryption. The app's second version is slated for release by the end of 2019 and will have an "action package" such as the ability to send useful notifications and reminders to users within a team. It will also allow for tracking instrument performance within a set range. In this way, a subtle drift upward, for instance, of temperature within the established range of temperature in an incubator, can be spotted before it becomes problematic. Such shifts can provide early warning of failure of many kinds of instruments. In addition, the app will be able to follow Westgard multi-rules to assist in determining the impact of fluctuations using a set of standards over time [8, 9].

Reflections™ finds utility in many different types of laboratories. The application has a sign-up and a sign-in section, including secure dual login, similar to banks. Once logged in, users can add one or more new labs and add members with specific assigned access levels. When a new lab is added, new instrument types (or groups) are defined by the user, followed by individual instruments within each group. For example, in the embryology laboratory, instrument groups could include different types of incubators, warmers, hoods, dewars, refrigerators, etc., and each group can include individual makes and models with different QC requirements. Within each instrument, parameters can be created and their acceptable normal ranges added. For example, for incubators, CO_2 and O_2 levels, pH, VOC, temperature, and humidity, display and measured, may be added, with specific acceptable ranges for each of these parameters determined, which can be modified at any time if needed without affecting the data already collected. When the instrument groups and instruments are in place, users can create one or more data logging plans, which can be set for desired intervals including daily, weekly, monthly, etc. These too can be edited later as desired. Data entry can be performed using any smartphone, tablet, notebook, or regular computer with Internet access, though handheld devices are preferable for their portability. Data review can take place at anytime from anywhere, which is particularly useful

for entities with multiple laboratories dispersed in different rooms, buildings, cities, or even countries. Directors and supervisors can check for task completeness and review data via their browser on any Internet-connected device including their cell phones. Summary reports can be generated and viewed by staff members with permitted access. These can be downloaded as PDF for printed records and emailed to overseers as required; alternatively, electronic signatures may be obtained. Fluctuation and detail reporting shows all entries made for instruments within any instrument type, so that instrument's recorded data can be seen for any time period as a graph, giving staff the opportunity to see early indications of instrument or system malfunction. Incident reporting allows for staff to enter important occurrences that merit further attention, reasonably divided into varying levels. This allows direct and effective follow-up according to urgency.

As far as the authors are aware, as of spring 2019, no other general applications are extant with such features. None of the existing utility apps offer the total flexibility that is allowed by Reflections™. None offer the useful reporting functions that allow accurate real-time instrument monitoring. There are applications suitable for certain business entities that have some similar features, but they have limited flexibility and often are enormously expensive to purchase and maintain. Reflections™ can be used by a range of business entities that require quality control and management, from ART laboratories to diagnostic laboratories, even zoos, chemistry labs, agricultural facilities, and wineries. Reflections™ provides a serious step toward the important but elusive goal of paperless quality management while improving service to patients and lowering cost.

Review Points

1. There are enormous advantages to electronic recordkeeping in ART. Though limitations related to clinical and laboratory practice variation among users exist, requiring customization at high cost, new and innovative ways of coding could overcome these limitations.
2. Only one of the three areas (EMR, enterprise, and QM) of ART clinic data handling has been addressed by commercially available electronic practice management products.
3. A first app for QC tracking of instruments has been recently introduced, but the field is in great need of a recordkeeping overhaul.

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Regulation of the ART Laboratory

Doris J. Baker

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Learning Objectives

- Describe methods of oversight for reproductive laboratories.
- Differentiate certification and accreditation.
- Describe major procedures performed in ART laboratories.
- Give reasons and cite examples for regulation of ART laboratory procedures differing among states.
- List concerns regarding ART laboratory procedures, and explain how the expertise of the embryologist might be responsible for these issues.
- Describe the current status of the clinical embryologist.
- List general criteria for certification for an embryologist and for an embryology laboratory director.
- Discuss future plans for recognition of the embryologist as a healthcare professional.

Assisted reproductive technology (ART) laboratories, also referred to as embryology laboratories, are a fundamental component of reproductive medical practices. Procedures performed in these facilities focus on the treatment of infertility but may also include processes for fertility preservation, sex selection, preimplantation genetic screening, and interventions for the prevention of genetic diseases. Professional organizations provide data on the status of ART worldwide [1–11]. The most recent triennial (2010–2013) account from the World Collaborative Report on Assisted Reproductive Technology's International Committee for Monitoring Assisted Reproductive Technology (ICMART) presented data between 58 and 61 countries from a total of nearly 2500 ART clinics [1]. Aggregate country data for the years 2008, 2009, and 2010 estimated that 4,461,309 ART cycles were initiated resulting in 1,144,858 live births. The number of oocyte aspirations increased by 6.4% between 2008 and 2010. Although ART application remained relatively constant for the time period (436 cycles/million in 2008 and 744 cycles/million population in 2010), there was a wide range among countries (8–4775 cycles/million population) worldwide. The most recent surveillance estimates that there are between 3706 and 3895 ART centers, an increase over the range of 3528–3877 previously reported in 2010 [2, 3]. The International Federation of Fertility Societies (IFFS) also monitors ART practices [4]. The latest IFFS Surveillance was reported in 2013 providing a view of ART applications, as they existed worldwide in the fall of 2012. This surveillance conveyed that countries with the highest number of ART facilities (≥200) were Brazil, China, India, Italy, Japan, and the United States and those with the lowest total (≤5) were Iceland, Ivory Coast, Latvia, the Philippines, Senegal, Slovenia, Togo, and Uruguay.

65.1 ART Interventions/Services

As with the usage of ART for treatments, the types of ART services that are performed in the various embryology settings differ greatly with variation among countries, within nations, and among national practices [1, 4]. The commonality is that ART services involve handling reproductive tissues in vitro for the purposes of establishing a pregnancy. Establishment of pregnancy may be limited to the use of participating couples' mature gametes although these gametes may be manipulated to improve rates of success. Spermatozoa may be injected into retrieved oocytes (intracytoplasmic sperm injection or ICSI), and in some laboratories, immature oocytes may be matured in vitro [12–14]. Third-party reproduction typically involves donated gametes and/or embryos to establish pregnancy [15–17]. In a limited number of cases, donor cytoplasm has been transferred to a recipient's oocyte [18]. Multiple-party reproduction may include gestational carriers or surrogates when the intended mother is unable to establish a pregnancy or cannot carry a gestation to term [19]. Posthumous insemination using postmortem spermatozoa also may be performed in ART laboratories [20].

Preimplantation genetic screening and diagnosis (PGS, PGD) may be performed on polar bodies or embryos for the prevention of disease with replacement of healthy embryos only [21–23]. Sex selection is performed for prevention of genetic disease. For example, in the case of a woman carrying the gene for an X-linked disorder that would result in disease in any male offspring, only non-affected female embryos would be transferred. This technique also may be used for sex selection based on preference of one sex over another for family planning [24]. In the forefront of practices for prevention of genetic diseases are mitochondrial replacement techniques (MRT) to prevent maternal transmission of mitochondrial disease from the affected woman to her offspring. The new techniques involve transferring either the nucleus from the intended mother's oocyte or both oocyte and spermatozoon pronuclei from a couple's recently fertilized oocyte into an enucleated donor oocyte containing healthy mitochondria. Offspring resulting from these procedures would have DNA from three different individuals [25–33].

65.2 Regulation and International Practices

The scope of ART procedures performed typically depends on regulation of the practice and the technology and expertise available at the facility. Regulation may be influenced by religion, culture, history, and politics (Box 65.1). Oversight of ART may be via statute, directive, licensing, guidelines, certification, and accreditation, or the ART laboratory and embryologists may function without benefit of any type of scrutiny [4]. A *regulation* is an authoritative rule dealing with

details or procedures that is issued by an executive authority or regulatory agency of a government, whereas a *statute* or *law* is enacted by legislature [34, 35]. For example, in the United States the Food and Drug Administration (FDA) has regulatory authority over cryopreserved reproductive tissues, and the Human Fertilisation and Embryology Authority (HFEA) regulates ART facilities via statute, the HFE Act 2008 [36, 37]. A *directive* is an official or authoritative instruction such as the EU Tissue and Cells Directive [38, 39]. A *license* is a permit from an authority [40]. The HFEA has responsibility for licensing all clinics and establishments in the UK that carry out IVF and human embryo research [36]. A *guideline* is a principle put forward to set standards or determine a course of action [41]. Guidelines for ART are typically set by a professional society but may be established by a government agency. In the United States, the American Society for Reproductive Medicine (ASRM) provides guidelines for embryology and andrology laboratories [42]. Although guidelines are typically voluntary, there are exceptions. Singapore voluntarily reports to the Director of Medical Services of the Ministry of Health, and in India, the state accrediting authority has the power to levy a fine for any violation and may even close a clinic for failure to comply with established guidelines [6, 43]. *Certification* is used for verifying that personnel have adequate credentials to practice certain disciplines, as well as for verifying that products meet certain requirements. Some form of external review, education, assessment, or audit often, but not always, provides this confirmation [44]. *Accreditation* is a specific organization's peer review process of a certification process [45]. In the United States, the Clinical Laboratory Improvement Amendments (CLIA) of 1988 regulations for clinical laboratories apply to andrology tests, and a voluntary peer review by the College of American Pathologists (CAP) accredits ART laboratories via their Reproductive Laboratory Accreditation Program [46, 47].

The 2013 IFFS Surveillance conveyed that regulation of ART varied among the 60 countries reporting with 31% having only legislation to regulate, 21% having only guidelines, and 37% having both legislation and guidelines. Nine percent of those responding to the survey had neither legislation nor guidelines. There was a licensing body to regulate ART in 74% of countries. Various methods for implementation were reported for countries with legislation: 16% had on-site inspections, 6% submitted a periodic report, and 29% had both inspection on-site and periodic reports. Two percent of respondents listed other methods, and 24% stated that no type of surveillance was undertaken [4].

Box 65.1 Glossary of Terms in Licensing and Regulation of the ART Laboratory

Accreditation—process that gives official authorization of approval by providing credentials that vouch for conforming to a standard
American Association of Bioanalysis—evaluates, through the certification process, individuals who wish to enter, continue, or advance in the clinical laboratory profession
American Board of Bioanalysis (ABB) Certification—based on an individual's education, experience, and knowledge of the laboratory field in which certification is granted
American College of Embryology (ACE)—professional organization for the advancement of the practice of clinical embryology
American Society for Reproductive Medicine (ASRM)—professional organization devoted to advancing knowledge and expertise in reproductive medicine and biology
Assisted hatching (AH)—in vitro procedure in which the zona pellucida of an embryo is thinned or perforated by chemical, mechanical, or laser methods to assist separation of the blastocyst
Assisted oocyte activation—a technique that simulates effect of spermatozoa penetration into an oocyte, inducing oocyte activation; used in combination with ICSI to improve fertilization outcome; typically recommended for cases when classic IVF has failed. Methods of activation include calcium ionophores, electrical stimulus, and ethanol
Assisted reproductive technology (ART)—all treatments or procedures that include the in vitro handling of both human oocytes and sperm, or embryos, for the purpose of establishing a pregnancy, including, but not limited to, in vitro fertilization (IVF) and embryo transfer, gamete intrafallopian transfer, zygote intrafallopian transfer, tubal embryo transfer, gamete and embryo cryopreservation, oocyte and embryo donation, and gestational surrogacy; does not include artificial insemination with either partner or donor sperm
Association of Clinical Embryologists (ACE)—professional body of and for embryologists in the United Kingdom, founded in 1993 to promote high standards of practice in clinical embryology and to support the professional interests of embryologists working in the United Kingdom
Board certified—having completed the process of board certification in a specialty field
Board examination—qualifying examination for a specialty field
Body of knowledge—represents the complete set of concepts, terms, and activities that make up a professional domain, as defined by the relevant professional organization and professional association

Cellular and tissue-based products (CGTPs)—established by Part 1271 by the FDA to establish good tissue practices to prevent introduction, transmission, and spread of communicable disease; refers to human cells or tissue intended for implantation, transplantation, infusion, or transfer into a human recipient which is regulated as a human cell, tissue, and cellular and tissue-based product or HCT/P; includes reproductive tissues

Centers for Disease Control and Prevention (CDC)—one of the major operating components of the Department of Health and Human Services (DHHS) whose mission is to collaborate to create the expertise, information, and tools that people and communities need to protect their health—through health promotion; prevention of disease, injury, and disability; and preparedness for new health threats

Centers for Medicare and Medicaid Services (CMS)—federal agency within the US Department of Health and Human Services (DHHS) that administers the Medicare program and works in partnership with state governments to administer Medicaid, the State Children's Health Insurance Program (SCHIP), and health insurance portability standards. In addition to these programs, the CMS has other responsibilities, including the administrative simplification standards from the Health Insurance Portability and Accountability Act of 1996 (HIPAA), quality standards in long-term care facilities through its survey and certification process, and clinical laboratory quality standards under the Clinical Laboratory Improvement Amendments

Certification—process through which an organization grants recognition to an individual, organization, process, service, or product that meets certain established criteria

Chimera—coexistence of more than one cell line in an individual, due to the fusion of originally separate zygotes

Clinical laboratory—any facility that does laboratory testing on specimens derived from humans to give information for the diagnosis, prevention, or treatment of disease or impairment and for the assessment of health

Clinical Laboratory Improvement Amendments (CLIA)—passed by Congress in 1988 to establish quality standards for all laboratory testing to ensure the accuracy, reliability, and timeliness of patient test results regardless of where the test was performed; financially managed by the CMS. The FDA is responsible for test categorization

College of American Pathologists (CAP)—medical society serving physician members and the laboratory community throughout the world; accredits laboratories; does accreditation of laboratories under deemed authority by CMS

Consanguinity—relationship by blood or by a common ancestor

Cryopreservation—freezing or vitrification and storage of gametes, zygotes, embryos, or gonadal tissue

Cytoplasmic transfer—ART procedure where the cytoplasm from a donor oocyte is injected into a recipient oocyte that has compromised mitochondria. A resulting embryo would have two sources of DNA: nuclear and mitochondrial from the recipient and mitochondrial DNA from the donor

Directive—order or instruction, especially one issued by a central authority

Directive 2004/23/EC—EU Tissues and Cells Directive adopted by the European Parliament, April 2004, that sets standards of quality and safety for the donation, procurement, testing, processing, preservation, storage, and distribution of human tissues and cells

Embryo—state of an organism's development that begins after the primitive streak develops and that persists until major organs are formed. In the human, the embryonic stage begins approximately 14 days postfertilization and encompasses the period when organs and organ systems are being formed or product of the division of the zygote to the end of the embryonic state, 8 weeks after fertilization (does not include parthenotes—generated through parthenogenesis—nor products of somatic cell nuclear transfer (SCNT)

Embryo donation—transfer of an embryo resulting from gametes (spermatozoa and oocytes) that did not originate from the recipient and her partner

Embryo/fetus reduction—procedure to reduce the number of viable embryos or fetuses in a multiple pregnancy

Embryologist—professional with specialized training in embryology; scientist having training and skills to handle oocytes, spermatozoa, and preembryos in the embryology laboratory

Embryology Laboratory Director (ELD)—certification by the American Board of Bioanalysis. Minimum requirements include a B.S. degree, having personally performed at least 60 ART procedures in humans under current standards of care, passing ABB's examination for ELD, and having directed an embryology laboratory prior to July 20, 1999

European Society of Human Reproduction and Embryology (ESHRE)—European professional organization with the main aim or promoting interest in, and understanding of, reproductive biology and medicine through facilitating research and subsequent dissemination of research findings in human reproduction and embryology to the general public, scientists, clinicians, and patient associations. It also works to inform politicians and policymakers throughout Europe and offers embryology certification

Ethics—a theory or a system of moral values

European Parliament of the Council—European Union's main decision-making body

Food and Drug Administration (FDA)—federal regulatory agency in the DHHS responsible for ensuring the safety of an array of consumer products. It has large scope, and it plays a critical role in a number of industries

Gamete intrafallopian transfer (GIFT)—ART procedure. Both sperm and oocyte are transferred to the fallopian tubes

Gestational carrier (*gestational surrogate*)—woman who carries a pregnancy with an agreement that she will give the offspring to the intended parent(s). Gametes can originate from the intended parent(s) and/or a third party (or parties)

Guideline—principle put forward to set standards or determine a course of action; typically does not carry the force of law

Health and Human Services (HHS)—department that is the US government's principal agency for protecting the health of the Americans and providing essential human services, especially for those who are least able to help themselves

High-order multiples—pregnancy or delivery with three or more fetuses or neonates

Human cells, tissues, and cellular and tissue-based products (HCT/Ps)—Center for Biologics Evaluation and Research (CBER), an FDA branch regulating HCT/Ps, including reproductive tissues, to prevent introduction, transmission, and spread of communicable disease by donor testing and following established good tissue practices under 21 CFR Parts 1270 and 1271, compliance effective May 2005

Human Fertilisation and Embryology Authority (HFEA)—UK's independent regulator that oversees the use of gametes and embryos in fertility treatment and research

Infertility—disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse

International Committee for Monitoring Assisted Reproductive Technology (ICMART)—independent, international, nonprofit organization that has taken a leading role in the development, collection, and dissemination of worldwide data on assisted reproductive technology (ART); International Federation of Fertility Societies (IFFS)

Intracytoplasmic sperm injection (ICSI)—procedure in which a single spermatozoon is injected into the oocyte cytoplasm

In vitro *fertilization* (IVF)—an ART procedure that involves extracorporeal fertilization

Law—body of rules of conduct established and enforced by an authority of legislation

Legal—meeting the requirements under law

Licensing body—competent authority able to grant permission to exercise a certain privilege that, without such authorization, would constitute an illegal act

Mandate—directive, order

Mitochondrial replacement techniques (MRT)—creating an embryo with nuclear DNA (nDNA) from the intended mother and mtDNA from a woman with a nonpathogenic mtDNA through modification of either an oocyte or zygote (fertilized oocyte) to prevent transmission of mtDNA disease from mother to child; two techniques: (1) maternal spindle transfer (MST), which involves manipulation of oocytes, and (2) pronuclear transfer (PNT), which involves manipulation of zygotes

National Health System (NHS)—comprehensive publically funded government healthcare system service in Britain covering virtually the entire population

Nuclear transfer—in ART, the introduction of the nucleus from the oocyte of an older patient into the enucleated oocyte of a young oocyte donor for the purpose of fertilization

Ovarian hyperstimulation syndrome (OHSS)—an exaggerated systemic response to ovarian stimulation characterized by a wide spectrum of clinical and laboratory manifestations; classified as mild, moderate, or severe according to the degree of abdominal distension, ovarian enlargement, and respiratory, hemodynamic, and metabolic complications. Severe OHSS is defined to occur when hospitalization is indicated

Oversight—supervision, control, overseeing, managing, administration

Preembryo—conceptus during early cleavage stages of development until approximately 14 days postfertilization

Preimplantation genetic diagnosis (PGD)—analysis of polar bodies, blastomeres, or trophectoderm from oocytes, zygotes, or embryos for detection of specific genetic, structural, and/or chromosomal alterations

Preimplantation genetic screening (PGS)—analysis of polar bodies, blastomeres, or trophectoderm from oocytes, zygotes, or embryos for detection of aneuploidy, mutation, and/or DNA rearrangement

Professional organization—a nonprofit organization seeking to further a particular profession, the interests of individuals engaged in that profession, and the public interest

Pronuclei—the haploid nucleus of a sperm or oocyte before fusion of the nuclei at fertilization

Public Law 102–493 (*Fertility Clinic Success Rate and Certification Act of 1992*)—requires assisted reproductive technology programs to report annually to the secretary of HHS, through the Centers for Disease Control and Prevention to report pregnancy success rates and report each embryo laboratory used by the program and whether it is certified and mandates development of model program for certification of embryo laboratories to be carried out by the individual states
Regulation—rule or order prescribed for management or government
Reproductive cloning—technology used to generate an animal that has the same nuclear DNA as another currently or previously existing animal, in a process called "somatic cell nuclear transfer" (SCNT)
Sanction—(n) mechanism of social control for enforcing a society's standards
Somatic cell nuclear transfer (SCNT)—transfers genetic material from the nucleus of a donor adult cell to an enucleated oocyte. The reconstructed oocyte containing the DNA from a donor cell must be treated with chemicals or electric current in order to stimulate cell division; once the cloned embryo reaches a suitable stage, it is transferred to the uterus of a female host where it continues to develop until birth
Standard—basis for comparison; a reference against which other things can be evaluated
Statute—a law enacted by a legislature
Therapeutic cloning—production of human embryos for use in research. The goal of this process is not to create cloned human beings but rather to harvest stem cells that can be used to study human development and to treat disease; stem cells are extracted from the inner cell mass of the blastocyst
Third-party reproduction—the use of oocytes, sperm, or embryos that have been donated by a third person (donor) to enable an infertile individual or couple, the intended recipient, to become parents
Total delivery rate with at least one live birth—estimated total number of deliveries with at least one live born baby resulting from the initiated or aspirated ART cycle including all fresh cycles and all frozen-thawed ART cycles. The rate is used when all of the embryos (fresh and/or frozen-thawed) have been used from one ART cycle. Delivery of singleton, twin, or other multiple pregnancy is registered as one delivery
Traditional surrogacy—treatment in which a woman is inseminated with sperm for the purpose of conceiving for an intended recipient. The surrogate has genetic and biological link to pregnancy she might carry
Values—important and enduring beliefs or ideals shared by the members of a culture about what is good or desirable and what is not
Vitrification—an ultrarapid cryopreservation method that prevents ice formation within the suspension, which is converted to a glass-like solid
Zygote—one-cell stage that follows breakdown of the pronuclear membrane and precedes the first cleavage or a diploid cell resulting from the fertilization of an oocyte by a spermatozoon, which subsequently divides to form an embryo
Zygote intrafallopian transfer (ZIFT)—procedure in which zygotes are transferred into the fallopian tube

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65.3 International Practices

Several IFFS 2013 survey questions addressed both the clinical practice and the role of the laboratory. The following tests, procedures, and services are lab associated.

65.3.1 IVF

The most basic ART procedure is in vitro fertilization and embryo transfer that resulted in the first in vitro birth in the United Kingdom in 1978 [48]. IVF is now performed in developed countries internationally. The procedure was unconstitutional in the country of Costa Rica until 2016 [49]. The lifting of the restriction ended the last full IVF ban in the world. Spin-offs from the IVF procedure such as gamete intrafallopian transfer (GIFT) and zygote intrafallopian transfer (ZIFT) are still practiced although GIFT appears to be limited to niche populations that may reflect religious dogma opposed to fertilization outside the body or to regulation [6, 50]. Embryo culture may be restricted to the cleavage stage or extended to the blastocyst stage of development [51].

65.3.2 Cryopreservation Procedures

Cryopreservation is routine in most settings but freezing may be limited to cleavage-stage embryos and partner semen to have available for backup in case of problems at the time spermatozoa are needed for insemination or may be expanded to include cryopreservation of zygotes, blastocysts, oocytes, donor spermatozoa, and ovarian and testicular tissues [52–55]. Cryopreservation procedures are dependent on the level of laboratory technology and on regulation. Newer technology, such as the technically simpler and rapid-to-perform vitrification procedures, has been responsible for the expan-

sion of cryopreservation in ART laboratories [56]. Moving cryopreservation of oocytes from the investigational status to an acceptable clinical procedure has also resulted in expansion of these services [4]. Regulation regarding preservation of embryos and gametes continues to be refined. Embryo cryopreservation is allowed by statute or guidelines in 83 countries. Venezuela is the only country to prohibit freezing fertilized oocytes, and Italy permits embryo freezing only in specific cases. Oocyte cryopreservation is permitted in 42 countries, and cryopreservation of germinal tissue is allowed in 70 countries but is not allowed in Ivory Coast and Senegal. There are often restrictions regarding cryopreservation of gametes, embryos, and reproductive tissues. For example, in Switzerland only zygotes may be cryopreserved. Fertility preservation is for cancer patients only in Australia, Hong Kong, Ireland, and Turkey, and in Spain it is limited to cases of high ovarian hyperstimulation syndrome (OHHS) risk [4].

65.3.3 Oocyte Maturation

Oocytes may be matured in vitro. In vitro maturation (IVM) is an evolving procedure that involves culturing immature oocytes to maturity in vitro to perform IVF. The procedure has the advantages of not requiring gonadotropin stimulation, making it less costly than traditional IVF, and eliminating the risks associated with patients at risk for ovarian hyperstimulation associated with pharmacologic gonadotropins. Oocytes can be retrieved at any time during the cycle, making the procedure ideal for young patients undergoing oncotherapy [4, 14]. ICSI is typically required for inseminating in vitro matured oocytes. The IFFS 2013 reported that 29 of 43 countries with statutes, laws, and guidelines permitted IVM, but two, Denmark and Senegal, prohibited the procedure. The practice was restricted by statute in 5 countries and by guidelines in 12. Nine countries reported using IVM as a routine part of their cryopreservation program [4].

65.3.4 Third-Party Reproduction

Gamete and embryo donation may be influenced not only by technology but also by history, culture, and predominant religion(s) in a state. The IFFS 2013 addressed this practice. Sperm donation is not allowed for non-IVF in 22% of the countries surveyed and is prohibited in the Arab nations of Egypt, Libya, Senegal, Tunisia, Saudi Arabia, and Turkey. Oocyte donation is not permitted in Austria, Italy, Norway, and Switzerland and is allowed only in special cases in Slovenia. Embryo donation is approved in 53% of countries responding to the 2013 IFFS survey but prohibited in many including Israel, Austria, Denmark, Iceland, Italy, Norway, Slovenia, Sweden, Switzerland, China, Egypt, Libya, Saudi Arabia, Senegal, Tunisia, and Turkey. Donation of germinal tissue is banned in 43% of reporting states [4].

Third-party reproduction may extend beyond gamete, embryo, and reproductive tissue donation to include gestational carriers and surrogates [19]. The term gestational carrier or gestational surrogate refers to an arrangement in which the gametes are of the genetic couple contracting the surrogacy arrangement with the surrogate host being unrelated to any offspring that might be born from the arrangement. This differs from a partial surrogacy where the surrogate is the genetic mother. The IFFS 2013 survey addressed gestational surrogacy only. Of the 62 responding countries, only 19 or 31% sanctioned the arrangement, and only 9 reported actually using gestational surrogates. States allowing the agreement had precise requisites regarding the contractual provisions [4].

65.3.5 Micromanipulation Procedures

65.3.5.1 Intracytoplasmic Transfer

Micromanipulation procedures, designed to increase the success of an IVF cycle, include ICSI and assisted hatching (AH) [12, 13, 57]. Although ICSI is indicated for male factor infertility, it is reportedly used routinely in ART units in several countries including Brazil, Australia, Ireland, Finland, Libya, the United States, South Africa, and Slovenia. The procedure is not prohibited in any state, and only Venezuela reports using the technology for specific cases only [4].

65.3.5.2 Assisted Hatching

Assisted hatching is a mechanical or chemical disruption of the zona pellucida to improve pregnancy rates. It may be performed on embryos with a thick zona pellucida, on those that have been frozen and thawed, and for women who are over 38 years of age or who have had repeated implantation failures. The technique also may be used to breach the zona pellucida prior to preimplantation genetic screening [4, 21, 22]. Assisted hatching is accepted in 58 of the reporting states. It is banned in Ivory Coast and Senegal and is used with restrictions in several countries [4].

65.3.5.3 Cytoplasmic Transfer

Cytoplasmic transfer involves the transfer of ooplasm from oocytes of young donors to the oocytes of older women to introduce healthy mitochondria believed to rescue an embryo from developmental block at the two-cell stage, referred to as the two-cell block [58, 59]. The procedure results in offspring with two different strains of mitochondrial DNA. Cytoplasmic transfer is permitted in Argentina, Greece, India, Kazakhstan, and the United Kingdom. It is not allowed in 25 countries, and law in Austria strictly prohibits the procedure. The FDA regulates the procedure in the United States. The use of the procedure requires an approval letter from the agency. To date, the FDA has not approved any applications. The procedure may be used for research purposes only in France [4, 26].

65.3.5.4 Mitochondrial Donation

In the forefront of micromanipulation procedures is mitochondrial donation, more technically accurately referred to as nuclear genome transfer, techniques designed to allow a small number of women with a rare kind of severe mitochondrial disease to have a healthy and mostly genetically related child. The techniques involve transferring the nucleus of an affected woman's oocyte, or the nucleus of a fertilized embryo, into a donor's enucleated oocyte or embryo, with the healthy mitochondria remaining. In this process, the nuclear DNA is inherited from the intended mother and father and mitochondrial DNA from the donor oocyte provider, resulting in offspring with DNA from three different individuals [25–33]. Great Britain was the first country to legalize mitochondrial transfer in 2015 [30]. In the United States, an FDA advisory panel says mitochondrial therapy research is ethical if limited to male embryos [33]. The agency is now considering mitochondrial replacement [25].

65.3.5.5 Genetic Screening and Diagnosis

Preimplantation genetic diagnosis (PGD) is more accurately referred to as preimplantation genetic screening (PGS) for determination of genetic disease. The procedure typically involves removing one or two blastomeres from an eight-cell embryo on day 3 of development for genetic analysis although screening can be performed using polar bodies and trophectoderm cells [22]. Only unaffected embryos are transferred. Of 40 countries with statutes, laws, and guidelines, 38 allow PGD. Two countries, the Philippines and Switzerland, do not allow the testing. In eight of these countries, PGD is restricted to specific hereditary disorders [4].

65.3.5.6 Sex Selection

Sex selection is applicable for family balancing or to prevent transmission of sex-linked, inherited genetic diseases. Selection may be by sperm sorting using flow cytometry to separate X- or Y-enriched semen for insemination, IVF following preimplantation genetic screening with selection of desired sex for transfer, or by other methods. Other methods consist of sperm separation on a gradient, timing of sexual intercourse, and termination of pregnancy with an unwanted sex. Sperm sorting selects for males with 75% success rates and for females with 85% success rates. IVF following PGS has a success rate of 99% [4]. IFFS 2013 shared that very few countries perform sex selection by either the sorting methods or IVF following embryo biopsy for determination of sex [4, 23]. The procedures are allowed in only 9 countries and are not allowed in 29. They were not mentioned by law in 5 but were reported as being practiced by one or both methods in 20 countries [4].

65.3.5.7 Posthumous Reproduction

To date, posthumous insemination has been performed using a man's cryopreserved spermatozoa or posthumous sperm retrieval [20]. The IFFS 2013 Surveillance queried respondents about this practice and learned that is was not permitted in most (43%) states [4].

65.3.6 Research

65.3.6.1 Research on the Preembryo

Research on the preembryo is controversial and based on religious principles and status of the conceptus. Of 54 responding to the IFFS question, 22 noted that experimentation is acceptable, 29 stated that it is unacceptable, and 3 did not know. Third-party approval was a requirement, and there was an established maximum age restriction, typically 14 days, beyond which experimentation was proscribed. The ruling tends to be in line with culture and religious dogma regarding when life begins [4].

65.3.6.2 Cloning

Therapeutic cloning involves harvesting stem cells from the human embryonic inner cell mass with the intent of having the cells undergo differentiation to be used for therapeutic purposes [60, 61]. Therapeutic cloning is used in 8 of the 46 countries that have laws, statutes, and guidelines and is not used in 38 countries. Research on embryonic stem cells is permitted in 14 of these countries [4].

Reproductive cloning or somatic cell nuclear transfer (SCNT) is a technique for cloning that involves removing the nucleus from a healthy oocyte and transferring DNA from the somatic cell of an animal to be copied. Following in vitro development, an early-stage embryo is implanted into a surrogate womb. The young animal, the clone, has the same genetic makeup as the animal that donated the somatic cell [60, 61]. Reproductive cloning in humans is prohibited in all countries except China, where there are no reports of the procedure being practiced [4].

65.4 Concerns Regarding ART Laboratory Practices

There are many concerns regarding ART practices, several of which are directly related to the laboratory and the embryologist. Embryologists with varying degrees of education and training preform the very advanced and technical ART procedures. ART procedures are associated with increased birth defects [62–85]. Whether the cause is procedural or due to the underlying infertility, or in some cases advanced parental age, confounds the problem. If procedural, there are also multiple factors to consider including culture media/conditions and the skill of the embryologist. Some ART-related birth defects and newborn health issues are due to higher order multiple births [63, 64, 66]. These births with their increased healthcare costs concern society as a whole [64, 80]. Although the clinician and the parents make the decision regarding the number of embryos to transfer, the quality of the embryos for transfer is directly related to the skill of the embryologist. The embryologist's capability determines whether the laboratory is successful with culture to the blastocyst stage, in vitro maturation of oocytes, cryopreservation of reproductive tissues, and successful embryo biopsy for

genetic screening. The skill of the technologist is crucial for performing micromanipulation procedures. ICSI does not allow for natural selection of spermatozoa and skips several stages in fertilization, including oocyte binding and penetration. The procedure is associated with several categories of birth defects, including epigenetic changes. It is not clear if the defects are due to the causal fertility problem, culture conditions, or the skillfulness of the embryologist [69]. Another micromanipulation, assisted hatching, can damage the embryo, making it unacceptable for uterine transfer [4, 21, 22]. Chimerism has resulted from IVF using ICSI and assisted hatching of the resulting embryo [78, 79, 82, 85]. Mitochondrial transfer may result in mitochondrial-nuclear mismatch and carryover of mutated mtDNA, affecting tissues of the child born from these techniques, and that would be passed on to the future generations [26].

Laboratory error is another adverse consequence of ART. Gametes have been switched, embryos have been damaged or lost, and embryos have been transferred to the wrong recipient [86–94]. Although these errors are rare, the results are devastating [87]. The greatest chance for error occurs in cryopreservation procedures due to the multiple steps involved in labeling, freezing, storage, and thawing gametes and embryos. The chance of error may increase if donor gametes and/or embryos are included in the procedures. Outcomes of medical negligence claims for 1 carrier for practices over a 10-year period (2005–2015) for 10 practices in 9 states for a total of 184,015 total IVF cycles were reported. There were 176 incidences resulting in 30 claims, of which 22 resulted in indemnity disbursements. Misdiagnosis or errors in diagnosis and lost oocytes or embryos continue to be the most frequent causes of claims [95].

Embryo biopsy, which demands much skill, is performed for the purpose of preimplantation genetic screening [96]. Improperly performed procedures may impact the developmental potential of the embryo. Nuclear transfer for prevention of mitochondrial genetic disease requires that the embryologist not transfer maternal DNA to the enucleated donor oocyte. Genetic material can be lost during transfer, or small amounts of mtDNA from the unhealthy oocyte may be transferred [26].

The embryologist must possess attributes in addition to advanced technical skills, including a commitment to medical ethics. Confidentiality is paramount in the ART setting, which may include patients wishing to maintain secrecy regarding situations such as their infertility or third-party reproduction.

65.5 Status of Embryology Profession and the Embryologist

Although ART has been an expanding discipline for more than 40 years, the field of embryology is not widely recognized, and the embryologists performing the numerous, highly complex, multidisciplinary procedures in ART facilities are not acknowledged professionally. International regulations and practices document this lack of recognition. Only 3 of 27 European healthcare systems recognize clinical embryology as an official profession [97]. In the United States, embryology laboratories, and embryologists, are specifically excluded from regulation that oversees clinical laboratories, and the Australian Health Practitioner Regulation Society Health Agency that enforces the Practitioner National Law (2009) regulates 14 health professions and has 39 protected titles; embryology is not among them [46, 98]. This lack of acknowledgment may reflect the fact that the field of clinical embryology is not cohesive in terms of (1) terminology describing the discipline and the embryologist, (2) regulation of the laboratory practice, (3) required education and training for embryologists, (4) certification, and (5) requirements for continuing professional development for these clinical professionals.

65.5.1 Terminology

Defining the embryology laboratory and the embryologist poses challenges. First, labeling the clinical embryology facility as a "laboratory" is problematic. For example, in the United States, CLIA defines a laboratory as a place "for examination of materials derived from the body (such as fluids, tissues or cells) for the purpose of providing information on diagnosis, prognosis, prevention, or treatment of disease" [99]. Because embryos are not derived from the human body, this definition does not match with standard clinical laboratory procedures. Efforts to include embryology laboratories under CLIA have failed because of the definition of facilities and testing that are under the CLIA mandate [100].

There is also absence of consistency regarding the status of the embryologist as determined by professional interest groups, legislation, and certifying groups. The lack of coherence prompted the Alpha Expert Panel Consensus Meeting in 2014, an international task force charged with arriving at a consensus on the professional status of the clinical embryologist [101]. To approach this question, the work group sent a questionnaire to 40 national and international societies for clinical embryology. Based on the 26 responses from groups representing 58 countries located in Africa, the Americas, Asia (including India), Europe, the Middle East, and Oceania, it was concluded that there was not an international agreement on the role of embryologists.

65.5.2 Regulation

Since laboratories are not always included in ART surveillance, the 2013 IFFS survey addressed laboratory surveillance separately and found that results were similar to data for clinical surveillance. The majority (77%) had some type of laboratory surveillance. Twenty-two percent had on-site inspections only, 8% periodic reports only, and 31% both on-site inspections and periodic reports. Sixteen percent reported that laboratory surveillance was by other methods.

Most (69%) centers were accredited, and 65% had laboratory certification. Voluntary accreditation via a national reproductive professional society was reported as a common method of laboratory assessment. Fifty-seven percent of reporting centers stated that specific penalties were in place for violations [4]. The Alpha survey reported that 19 of 27 respondents favored regulation with some noting that regulation was in progress [101].

65.5.3 Education and Training

Acceptable education and training for clinical embryologists vary according to state. Although some have minimal requirements for embryologists, most legislation and accrediting bodies rely on the embryology laboratory director to determine education qualifications and training for reporting embryologists. Expectations for laboratory director include an earned doctorate in science although a master of science is acceptable in some countries. Most employers require only a bachelor's degree for embryologists entering the field [97]. A BSc/MLT was reported as the minimum requirement for embryologists for countries with regulations or published guidelines [101]. Certification programs do, however, have education requirements.

Typically, senior-level colleagues train new embryologists. Accrediting agencies typically require documentation of education and training, and these records are compulsory for application for embryology certification.

Lack of curricular-based programs in the field may contribute to the varying educational requirements for embryologists. There are a limited number of programs awarding M.S. and Ph.D. degrees in clinical embryology. These programs are located primarily in Europe [102]. The value of degree programs in clinical embryology continues to be challenged. Several programs have opened and closed in several countries over the decades. An established program in the United States, which had graduated several successful embryologists from certificate and master's programs over an 11-year period and which had opened a basic sciences Ph.D. with clinical rotations in andrology and embryology, was closed to support more traditional medical programs [103, 104].

The Association of Clinical Embryologists (ACE) Training Committee that administered and awarded a Certificate in Clinical Embryology stopped accepting applications as of December 31, 2015 [105]. Qualifications for the certificate were based on a minimum of BSc degree, current employment as a clinical scientist, supervision by an "in-house" HCPC-registered clinical scientist, completion of a log book documenting candidate's ability to perform various procedures in assisted reproduction, and a final examination. The course was expected to take approximately 2 years to complete.

Education programs that are ongoing are not consistent in terms of didactic curriculum and required clinical training and/or experience. Established programs for educating and training other health professionals follow a model that dictates certain courses and clinical experiences. For example, in order to be accredited as a medical laboratory science program in the United States, the curriculum must include a minimum number of credits in both lectures and student laboratories for all applicable discipline areas (clinical chemistry, hematology, immunology, microbiology, management) followed by clinical practica in all areas. The degree is awarded following completion of coursework and clinical practica [106].

65.5.4 Certification

Certification is not required in most states. The Alpha survey found that only 5 of 27 respondents required this credential. An education survey of centers in Australia and New Zealand conducted by the Fertility Society of Australia in 2010 addressed certification. They reported that although 60% of respondents wanted certification, 10% did not, and 30% were not sure [101].

Eligibility for embryology certification is based on education credentials, documentation of successful performance of clinical embryology procedures, and passing applicable examinations. This method of credentialing is not in line with required education and certification for other clinical laboratory personnel. In the United States, medical laboratory scientists (also referred to as medical technologists and clinical laboratory scientists) must complete a degree specific for the field and pass a national qualifying examination prior to entry into the field. As opposed to work experience being a requirement for certification, laboratory practice is a requisite for specialty certification in a specific clinical laboratory discipline, such as hematology or clinical chemistry [107].

The European Society for Human Reproduction and Embryology (ESHRE) provides the only internationally recognized certification [108]. Certification as a clinical embryologist requires a BSc in natural sciences and a minimum of 3 years hands-on experience with human gametes and embryos in a clinical ART laboratory, documented in a submitted logbook, and successful completion of a certifying examination. To qualify as a senior clinical embryologist, the individual must hold an MSc or Ph.D. in natural sciences and be able to document a minimum of 6 years' experience.

In the United States, the only embryology certification that is recognized by the embryology laboratory accrediting agencies (College of American Pathologists or CAP and the Joint Commission) and state licensing boards that certifies individuals in embryology is offered by the American Board of Bioanalysis (ABB) [109–111]. To be certified as an embryology laboratory scientist, an individual must document 30 ART laboratory procedures in humans and 1 year of acceptable full-time experience in the discipline of embryology, meet minimum education requirements, and pass examinations in basic laboratory knowledge and embryology. Minimum education accepted is 24 h semester hours of

courses in science or medical laboratory technology. Although an embryologist directing a laboratory prior to 1999 may grandfather as a director with minimum education requirements, individuals becoming directors after 1999 must meet stringent requirements. They must have an earned doctorate degree with chemical, physical, biological, or clinical laboratory science as a major and have completed a minimum of 32 semester hours in these sciences and have a minimum of 4 years of clinical laboratory training or experience within the 10 years immediately prior to the application date on human specimens, or both, including at least 2 years of experience within the 10 years immediately prior to the application date directing or supervising high-complexity testing in a clinical setting. In addition to these general requirements for laboratory director, the individual must have experience including 60 personally performed, completed assisted reproductive procedures and pass the ABB examination in general knowledge and at least 1 of the following disciplines or specialties: andrology, chemistry (including urinalysis, endocrinology, and toxicology), diagnostic immunology, embryology, hematology (including flow cytometry), microbiology (includes bacteriology, parasitology, virology, and mycology), molecular diagnostics, or public health microbiology.

65.5.5 Continuing Professional Development (CPD)

Continuing professional development (CPD) refers to continuing education to maintain knowledge and skills, following initial training. CPD is typically a regulatory requirement or guidance. For example, the European Union Tissues and Cells Directive (2004/23/EC—Article 18) states that "The centre should establish documented procedures for staff management that ensure all staff have initial basic training and updated training as required, on-going competence assessment, continuing education and professional development, and appropriate access to meetings and communications" [112]. However, there is a lack of this expectation internationally. Only three countries in the Alpha survey reported mandatory, regulated CPD, and two others had voluntary, regulated systems in place. One had a requirement for CPD, but no regulation in place, while four were planning to have national CPD systems [101].

Certifying bodies, including those for embryology, require CPD for maintenance of certification. ESHRE offers the opportunity to revalidate certificates through the Continuous Embryology Education Credit (CEEC) system [113]. Credits must relate to the field of embryology and assisted conception. Attendance at scientific meetings, publications, contributions to meetings, and courses will all be taken into consideration for the award of credits. Senior clinical embryologists will need ten credits over a 3-year block, and clinical embryologists must have six credits, obtained over a 3-year period, in order to apply for renewal of their certificate. The AAB also requires continuing education for renewal [114]. Certificants must document at least 2.4 CEUs (24 contact hours) each biennium. Acceptable credits are stricter than those of ESHRE. For example, all publications are not acknowledged.

65.6 Future

The range of disciplines associated with the multidisciplinary field of ART continues to evolve. New procedures are accompanied by the potential for genetic, social, and legal issues. For example, cryopreservation of oocytes and reproductive tissues opens the door for posthumous reproduction beyond the use of spermatozoa, along with issues regarding custody of gametes and tissues [4, 52–55]. Mitochondrial transfer with associated genetic implications is legal in Great Britain and is being reviewed in other countries. Polar body genome transfer and maternal spindle transfer are on the horizon [26]. Assisted oocyte activation is undertaken in some ART facilities [115]. Cloning, though not practiced, is legal in China [4]. It is anticipated that these advances coupled with malpractice claims and harsher violations will result in updates or increases in regulations. The 2013 IFFS reported that legislation had been updated in 43% of countries surveyed since the 2009 report and that 77% considered the new regulations an improvement [4].

Recognition of the field of embryology and the professional status of the embryologist is expected. As the field advances, the embryologist will assume greater responsibilities. The importance of the embryologist's credentials is highlighted by Great Britain's requirement for mitochondrial transfer. As part of the license to perform the procedure, *the competence of the embryologist must provide a CV with references that support their experience and knowledge and documentation of competence in performing micromanipulation on oocytes or embryos* [116]. The demand for education for embryologists is being realized. Professional societies and individual practices continue to offer numerous hands-on courses in specific techniques.

A newly opened Ph.D. program in reproductive clinical science focuses on education of embryologists already practicing in the field [117]. It is an online program that has been designed "for adult learners and working professionals who are in the workforce, while they are enrolled in this program."

Although a limited number of degree programs are available, they are not part of an overarching system that confers consistency and continuity to the academic programs. Degree programs in clinical embryology are not consistent in terms of coursework and clinical experience. This is not in line with other professional programs that have set requirements for courses and clinical practica prior to taking a certification/license examination [107]. This was addressed by the consensus work group resulting in Alpha's plans to develop an educational program for clinical embryologists. The planned program "will provide the material to meet the consensus requirements for knowledge development, as well as competency-based training, and continuing professional

development, to meet the current and future needs of Clinical Embryologists worldwide" [101]. This important first step will give them parity with other healthcare professionals. This is a critical step in positioning embryology to function as an independent profession and having embryologists recognized as a healthcare profession. Once achieved, these professionals will be able to establish guidelines, develop criteria for education and continuing education, and, most importantly, be able to influence policy regarding their practice.

Review Questions

1. Based on the most recent reports, how many live births have been produced using ART procedures?
2. Distinguish between regulation, statute, directive, and license.
3. Define laboratory certification and accreditation.
4. List at least five countries in which a specific ART procedure is forbidden. Associate this ban/restriction with the underlying reason(s).
5. List three methods for sex selection.
6. Describe assisted oocyte activation.
7. What are the major concerns regarding cytoplasmic and mitochondrial donation?
8. How do therapeutic and reproductive cloning differ? In what country is reproductive cloning legal?
9. What are the most common errors in the ART laboratory? How might these errors be minimized?
10. What tends to be the minimal education requirement for a clinical embryology director? An entry-level embryologist or embryology assistant?
11. What qualifications do the certifying bodies ESHRE and ABB require for embryology certification?

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Legislation in the UK

Rachel Cutting

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Learning Objectives

- To provide an overview of the history of legislation of ART in the UK
- To explain the structure and functions of the HFEA
- To describe the legislatory framework that centres must work within
- To discuss how centres are assessed for compliance and describe the process of licensing of centres by the HFEA

66.1 History of Legislation in the UK

66.1.1 The Warnock Committee

Although the birth of Louise Brown in 1978 was a major breakthrough in the treatment of the infertile couple [1], it raised many ethical concerns with both professionals and the general public. In response, a committee was established in 1982 in the UK to inquire into the technologies of in vitro fertilisation (IVF) and embryology and to form the principles of future regulation. Chaired by Mary Warnock, the terms of reference were to 'consider recent and potential developments in medicine and science related to human fertilisation and embryology; to consider what policies and safeguards should be applied, including consideration of the social, ethical and legal implications of these developments; and to make recommendations' [2]. The findings, which considered all processes involved in IVF, donor insemination and surrogacy, were published in the Warnock Report in 1984. The report concluded that the human embryo should be protected by law by giving it a special status outside the human body; however, research of human embryos could be permitted under certain circumstances. Other recommendations included the provision of counselling and ensuring patients give informed consent prior to treatment commencing. A key proposal was to establish an authority to regulate both research and treatment [2]. The first step to meeting the 60-plus recommendations in the report was for the Medical Research Council and the Royal College of Obstetricians and Gynaecologists to establish an interim licensing authority to provide a framework for inspection.

66.1.2 Founding the HFEA and the HFE Act 1990

Following the Warnock Report, the government undertook an extensive public consultation. The findings led to the publication of a government white paper, 'Human Fertilisation and Embryology: A Framework for Legislation', in 1987. The act drafted following this paper received royal assent on 1 November 1990 and paved the way for the establishment of the Human Fertilisation and Embryology Authority (HFEA) in 1991. This executive, nondepartmental public body was the first statutory body in the world and had the remit under the legislation to regulate IVF clinics through the licensing of:

- The creation of human embryos outside the body and their use in treatment and research
- The use of donated gametes and embryos
- The storage of gametes and embryos

The HFEA is comprised of a chair and members. The members who are appointed via the Appointments Commission are from diverse backgrounds and have the scope to determine policies and review treatment and research licence applications. Several committees support the process by providing advice on specific aspects, for example, the Audit and Governance Committee oversees corporate governance, risk, audit arrangements and financial matters and the Scientific and Clinical Advances Committee reviews and provides recommendations on recent developments. The HFEA is also obliged to provide information to the public, particularly those having treatment and donating gametes or embryos or those who are donor conceived. For this process to work efficiently, the HFEA maintains a formal register of information which records all treatments and donor information. The information held on this register can be used for research purposes depending on the type of consent provided by the patient.

66.1.3 Changes in Legislation 1991–2016

Although the 1990 legislation remained in force until the amended act in 2008, there have been various changes to the UK regulations over the years. This included allowing the storage periods for embryos to be extended in certain circumstances in 1996 and extending the purposes for which embryos could be used in research to include 'increasing knowledge about the development of embryos, increasing knowledge about serious disease and enabling any such knowledge to be applied in developing treatments for serious disease' in 2001.

A major change which impacted greatly on centres was the removal of anonymity from gamete and embryo donation which came into force in April 2005. This change permitted donor-conceived children access to the identity of their sperm, egg or embryo donor upon reaching the age of 18. This change raised concerns regarding the impact of this on donor recruitment, but although initially studies showed an overall trend in a decrease in recruitment, other studies have reported that over a longer time period, they were able to meet demand for recipient cycles [3]. At this time, payment for donation was not permitted, so in 2010 the HFEA started to review the legal and ethical issues surrounding payment and undertook a public consultation to formulate a new policy which came into force on 1 April 2012 [4]. This policy allows centres to compensate egg donors £750 per cycle and sperm donors £35 per centre visit with the flexibility for a donor to claim more if higher expenses are incurred, and receipts are produced as evidence.

In 2004, new EU regulations regarding common safety and quality standards were introduced to enable easier movement of tissues between members of the EU. UK centres had until 2007 to comply with the introduction of Directive

2004/23/EC [5] and the Commission Directives 2006/17/EC [6] and 2006/86/EC [7]. The HFEA was nominated as the competent body to assess centre compliance. The directives encompassed donation, procurement, testing, processing, preservation, storage and distribution of human tissue and cells.

Technology advances rapidly in assisted reproduction technology (ART), and in 2004, it became apparent that scientific developments and changes in public attitude necessitated a review of the 1990 Act. After public consultation and a review by the House of Commons Science and Technology Committee, the government published the white paper [8]. Following this, the draft bill was published in 2007, and after scrutiny the new bill received royal assent in November 2008.

The HFE Act 2008 [9] is divided into three parts:

1. Amendments to the Human Fertilisation and Embryology Act 1990
2. Parenthood
3. Miscellaneous and general

The main new elements of the 2008 Act [9] are:

- Ensuring that the creation and use of all human embryos outside the body – whatever the process used in their creation – are subject to regulation
- A ban on selecting the sex of offspring for social reasons
- Removal of the previous requirement within 'welfare of the child' of the child's 'need for a father'
- Allowing for the recognition of both partners in a same-sex relationship as legal parents of children conceived through the use of donated sperm, eggs or embryos
- Enabling people in same-sex relationships and unmarried couples to apply for an order allowing for them to be treated as the parents of a child born using a surrogate
- Changing restrictions on the use of data collected by the HFEA to make it easier to conduct research using this information
- Provisions clarifying the scope of legitimate embryo research activities, including regulation of 'human admixed embryos' (embryos combining both human and animal material)

Statutory storage limits for gametes and embryos increased within the new act from 5 to 10 years. It also became possible to extend the storage of gametes and embryos up to a maximum of 55 years provided that at each 10-year checkpoint, the centre obtains a written opinion from a registered medical practitioner that the person to be treated (or one of the people who provided the eggs or sperm) is or is likely to become prematurely infertile.

66.2 Code of Practice

Section 25 of the 1990 Act requires the HFEA to maintain a code of practice to provide guidance to centres in order for them to comply with the regulatory requirements when providing licensable activities. The first edition was published in 1991; the latest edition, the ninth edition, was published in January 2019. The code comprises of 33 guidance notes, each one describing the mandatory requirements, the HFEA interpretation of the mandatory requirements, best practice guidance and other relevant legislation, professional guidelines and information [10]. This comprehensive document is well used by centres and forms the basis of any procedures and processes which are carried out in a licensed centre. The 33 guidance notes are as follows:

1. Person responsible
2. Staff
3. Counselling and patient support
4. Information to be provided prior to consent
5. Consent to treatment, storage, donation, training and disclosure of information
6. Legal parenthood
7. Multiple births
8. Welfare of the child
9. Preimplantation genetic screening (PGS)
10. Embryo testing and sex selection
11. Donor recruitment, assessment and screening
12. Egg sharing arrangements
13. Payments for donors
14. Surrogacy
15. Procuring, processing and transporting gametes and embryos
16. Imports and exports
17. Storage of gametes and embryos
18. Witnessing and assuring patient and donor identification
19. Traceability
20. Donor-assisted conception
21. Intra-cytoplasmic sperm injection (ICSI)
22. Research and training
23. The quality management system
24. Third-party agreements
25. Premises, practices and facilities
26. Equipment and materials
27. Adverse incidents
28. Complaints
29. Treating people fairly
30. Confidentiality and privacy
31. Record-keeping and document control
32. Obligations and reporting requirements of centres
33. Mitochondrial donation

Centres use the Code of Practice to check their own compliance and for reference for many different clinical situations. In recent years, there has been much focus towards witnessing and minimising multiple births. The implications of inadvertent mixing of gametes or embryos resulting in the birth of a child with the 'wrong' genetic parents are catastrophic. It is therefore a mandatory requirement that centres have robust contemporaneous witnessing procedures and documentation in place to double check both the identity of patients and their samples throughout the treatment pathway. Electronic witnessing can be used, but a full risk

assessment must be conducted, and certain conditions such as double manual witnessing must occur when gametes are inseminated at embryo transfer or when samples are put into or removed from storage.

An expert report published in 2006 [11], which highlighted that multiple pregnancy was the biggest risk to the health of the mother and child during ART, led to the HFEA producing a policy and integrating the requirement for a multiple birth minimisation plan into the Code of Practice. The policy adopted a stepwise approach with the target being reduced over a period of 4 years. Centres are currently obliged to ensure their multiple birth rates do not exceed 10% and to perform regular audits to evaluate the effectiveness of their policies. Although after a legal challenge the licence condition (T123) had be to be removed, the HFEA remains committed to reducing multiple birth rates, and centres who do not meet the target are invited to attend a management review at the HFEA to discuss the issues.

The most recent Code of Practice focusses on the leadership role of the person responsible and introduces the concept of a patient support policy which ensures centres provide emotional support to patients before, during and after treatment.

66.2.1 Licensing and Inspection of UK Centres

In order to carry out assisted reproduction techniques in the UK, centres must be inspected by the HFEA to assess compliance against the HFE Act 1990 (as amended). The compliance cycle is 4 years with an inspection within every 2 years. An initial or renewal inspection has a planned agenda and is usually conducted with between two and four inspectors over 1–3 days depending on the size of the unit. Prior to the inspection, centres are asked to complete a self-assessment questionnaire which is analysed by a risk tool alongside other general performance measures. The focus of inspection is always on the quality and safety of patient care and protection of the embryo. Inspectors will directly observe practice, interview patients and check records. An interim inspection is often unannounced and will be focussed around specific themes. More frequent targeted inspections may be carried out if specific concerns are raised. In 2013, the HFEA extended its remit to include the inspection of surgical procedures in England to prevent centres being inspected by the Care Quality Commission (CQC) as well as the HFEA. This helped to reduce regulatory burden on centres and to streamline the regulatory process.

Once an inspection is complete, a report is produced, and the licensing decision whether to grant a licence is made by the licensing committee. Clinics do hold the right to appeal through the Appeals Committee. The HFEA also continually monitors a centre's performance through a risk tool which focusses on six key areas: incorrect identification of gametes or embryos; cross infection of gametes, embryos or patients; consent failures; damage or loss of gametes or embryos; multiple pregnancy; or incorrect or incomplete information on donors. The system also analyses a centre's success rates, multiple pregnancy rates, submission of critical donor information, payment of fees and incident reporting. Reporting incidents is a statutory requirement. Incidents are defined by the HFEA as 'any event, circumstance, activity or action which has caused, or has been identified as potentially causing harm, or loss or damage to patients, their embryos and/or gametes, or to staff or a licenced centre, including serious adverse events and serious adverse reactions'. Once received, the HFEA will grade the severity of an incident and determine what further action is required.

If a centre is non-compliant and there is a risk to patients, donors, gametes or embryos, then a formal process commences through the executive licensing panel using the guidance from the compliance and enforcement policy. Ultimately, a licence can be revoked.

66.2.2 Recent Changes

Although in 2015 the UK parliament voted to permit the use of mitochondrial donation for the purpose of avoiding the inheritance of severe mitochondrial disease, the expert panel reporting to the HFEA recommended that the safety and efficacy of the technique required further evaluation. The further 2016 review [12] provided comprehensive evidence in order for the HFEA to determine whether the technique could be used in clinical practice. The report concluded that clinical treatment could proceed cautiously in restricted circumstances with further research being conducted. On 15 December 2016, the HFEA permitted the use of this technology.

Section 33 in the Code of Practice outlines the specific criteria which must be complied with for treatment to be undertaken. Individual cases of maternal spindle transfer (MST) or pronuclear transfer (PNT) have to be submitted to the HFEA's Statutory Approvals Committee for authorisation.

66.2.3 Other Accreditation Schemes

Many clinics seek to optimise their services and comply with the HFEA requirement to have a demonstrable quality management system by choosing to be ISO9001:2015 certified. Diagnostic andrology services must also be certified to ISO15189:2012 or equivalent. The professional bodies relating to reproductive medicine produce best practice guidelines and provide comprehensive training and validation programs. In the UK, embryologists are trained via the master's-level Scientist Training Program in order to attain state registration.

66.3 Conclusion

The HFEA provides a unique comprehensive regulatory mechanism for ART. The framework drives standards and ensures high-quality patient care. Although seen by some as

burdensome, the regulations provide protection to both patients and embryos, and they have served to reassure the patient that this sensitive and ethically challenging area of medical practice is performed ethically.

Review Questions

1. Following the Warnock Report, the HFE Act received royal assent on 1 November 1990 and paved the way for the establishment of the Human Fertilisation and Embryology Authority (HFEA) in 1991. The HFEA regulates IVF clinics through the licensing of?

2. What major change in legislation in 2005 had impacted on donor treatments, and what further change in 2012 was made to help encourage people to come forward to donate?

3. The HFEA licences centres through an inspection process; describe the inspection cycle.

4. What are the current statutory storage periods for gametes and embryos, and under what circumstances can the storage period be extended?

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Regulation, Licensing, and Accreditation of the ART Laboratory in Europe

Julius Hreinsson and Borut Kovačič

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Learning Objectives

This chapter aims at informing the reader of the following:

- Overarching regulatory framework in the field of assisted reproduction in the European Union with special emphasis on the EU Tissues and Cells Directive (EUTCD)
- Relevant European institutions in this respect
- Important differences in national legislation among European countries
- Status of clinical embryology in Europe

67.1 Introduction

Legislation on ART varies considerably in Europe. Rules and regulations regarding procedures such as gamete donation, embryo cryopreservation, and other aspects of ART are not identical among the European countries. Details regarding specific requirements for laboratories vary as well; examples of this are the requirement of ISO 15189 accreditation of ART laboratories in some countries and ISO 9001 certification in several other countries. We will first discuss relevant European institutions in health care which influence the practice of ART and standards relevant in Europe. After that, we will focus on common requirements with reference to the EU Tissues and Cells Directive (EUTCD) [1], and at the end of the chapter, we will discuss the great variation in national legislation among the European countries.

Although some laboratories in some European countries may currently be operating without fulfilling the specifics in the EUTC Directive, they will in the coming years be expected to do so. This chapter is not planned to give guidelines as to how to perform the various ART procedures in the clinic or laboratory. For this purpose, other documents have been published, for example, De los Santos et al. [2].

The terms regulation and licensing may be considered relatively unequivocal. The term accreditation on the other hand occasionally gives rise to potential misinterpretations in the context of medical laboratories. It may be understood to mean recognized conformity to the international ISO 15189 standard on the one hand, or it may refer to the evaluation of competence by a designated authority without necessarily adhering to any particular ISO standard. In this chapter, we are referring to the second interpretation unless otherwise specified.

The most commonly applied international standards in the IVF laboratory are the ISO quality control standards, published by the International Organization for Standardization (ISO) (► www.iso.org). The two most commonly applied ISO standards are ISO 9001 for certification of quality management systems and ISO 15189 for accreditation of medical laboratories. Many laboratories also apply the ISO 14001 standard for environmental management system. In addition to the ISO standards, standards for good medical/laboratory practice (GMP/GLP) are also in use.

Common to these standards are stringent rules on document control as well as a requirement for a thorough description of all methods and procedures in use in the operation at hand. There are certain differences between the standards, where ISO 9001 places emphasis on process analysis and continuous improvement. ISO 15189 on the other hand focuses more on laboratory competence and the competence of personnel active in the laboratory. Both these standards are applicable to the IVF laboratory, and implementing them will greatly improve the quality in the laboratory. One great advantage of the ISO 9001 standard is that it can be applied to the laboratory and clinic alike which simplifies the implementation of the quality control routines and emphasizes the cooperation between the laboratory and the clinic.

67.2 European Institutions in Health Care

Even though every European country has its governmental institutions, taking care of its health-care system, there are also European institutions involved in creating health-care politics. The Council of Europe, founded in 1949 located in Strasbourg (France), is one of them and now consists of 47 member states. Its main task is promoting intergovernmental cooperation among all European countries to improve the quality of life for all Europeans not only in the areas of public health but also of human rights, democracy, rules of law, and culture.

Rapid progress of medicine makes it necessary for the fields of human rights, religion, and law to adjust to it. The same level of public health development is not expected due to economical, social, religious, and political differences among European countries. However, the main role of the Council of Europe is to harmonize European health-care systems and their ethical issues related to the rapid advances in biological and medicinal research and to provide minimal standards for all national health-care systems. Transplantational medicine with its rapid development is a good example of how necessary the coordination among the European countries is. Human tissues and cells are being used in an increasing variety of new ways, and today the transplantation therapy and assisted reproduction techniques are already routine methods used in daily practice. However, using human tissues and cells also raises questions of safety, quality, efficacy, and ethical dilemmas.

67.2.1 European Committee on Organ Transplantation

In 1987, the Council of Europe Committee of Experts on the Organizational Aspects of Cooperation in Organ Transplantation was created and later formed the secretariat responsible for activities related to organs, tissues, and cells. This secretariat was transferred to the European Directorate for the Quality of Medicines and Health Care (EDQM) of the Council of Europe.

67.2.2 European Directorate for the Quality of Medicines and Health Care

Today, the EDQM consists of internationally recognized experts from the Council of Europe member states, observer countries, the European Commission, representatives from the Committee on Bioethics of the Council of Europe, the WHO, and different nongovernmental organizations. The EDQM is a key European organization involved in harmonization, coordination, standardization, regulation, and quality control of medicines, blood transfusion, organ transplantation, pharmaceuticals, pharmaceutical care, consumer health, cosmetics, and food packaging. It promotes the noncommercialization of organ, tissue, and cell donation and develops ethical, quality, and safety standards.

The EDQM/Council of Europe has established several recommendations in the field of transplantation covering the ethical, social, scientific, and training aspects. Whereas agreements and conventions are binding of the states to ratify them, recommendations and resolutions are statements to governments that propose actions to be followed.

Article 168 of the Treaty on the Functioning of the European Union, which is the article about public health in the European Union (EU), gives the EU a mandate to establish high-quality and safety standards for substances of human origin, such as blood, organs, tissues, and cells with the aim to provide the common approach to the regulation of human tissues and cells across Europe [3]. As a result of this mandate, the European Parliament and the Council issued the Directive 2004/23/EC, the first of the EU Tissue and Cells Directives [1], followed 2 years later with two new directives, the Commission Directive 2006/17/EC [1.B] and the Commission Directive 2006/86/EC [1.C].

The EDQM/Council of Europe also issues guidelines. One of these is the second edition (the third edition is in preparation) of the guide to the quality and safety of tissues and cells for human application [4], including also a special chapter about ART. These guidelines help in understanding the EU Tissue and Cells Directives in detail. They are mostly written very generally with the aim to cover all fields of medicine that use live human tissues and cells for treatments.

67.3 The EU Tissue Directive and ART

The tissue directive was conceived to provide a regulatory framework for the foreseen rapid development of regenerative medicine. It was immediately obvious that other areas of medicine, such as transplantation medicine and assisted reproduction, were conceptually related and therefore needed to be covered by any regulatory initiatives. However, stem cell culture, organ transplantation, and assisted reproduction present very different challenges and have requirements that may be in conflict if a too rigid legislation is applied. The EU consulted with experts in the respective areas, and the technical directives, which guide the implementation of the tissues directive, are a result of the efforts to bring the different perspectives together. However, more work needs to be done to adapt the overall objectives of the directive to the practicalities of assisted reproduction. This chapter describes the current interpretation of the tissues directive for implementation in assisted reproduction.

67.3.1 Laboratory Personnel

Strong but not specific requirements for competence of personnel, both formal and practical, are put forward by the EU directive. The clinical disciplines such as medicine and nursing already have formally recognized education and training systems. The relevant authorities in each country issue formal license to practice, and separate licensing often does not follow formal training in ART. In spite of this, formal training programs for gynecologists are available (ESHRE/EBCOG). Formal training programs for embryologists however are not easily available in most European countries. Usually, some form of biomedical or medical-technical education is required as a basic education for the embryologist, but the complicated micromanipulative nature and richness of detail in the work as well as its academic nature places high demands on the embryologist.

In response to this, the European Society of Human Reproduction and Embryology (ESHRE) has established a certification program for clinical embryologists where an academic level of competence is established. Practical training still has to be performed at the clinics. This certification program forms an excellent basis for acknowledgement of the embryologist's competence in years to come.

In the EU directive, many of the details regarding which level of competence should be attained by the various categories of personnel are left for the individual clinics to decide. The clinic in question has to document in its quality system, and enforce in practice, how sufficient competence of personnel is regulated with reference to the activities performed. The following requirements are put forward by the directive:

- Evidence of training and competence testing must be documented. Personnel must be assessed regularly for competence, and professional development must be ensured.
- Sufficient numbers of personnel at the clinics must be ensured.
- Knowledge of the legal framework and ethical aspects must be established.
- The clinical disciplines have established licensing systems in all countries, but each laboratory must ensure the competence of embryologists employed at the clinic/lab.
- Laboratory and clinical directors must have documented academical and practical experience for several years.
- Access to the laboratory of a senior medical consultant is mandatory under the EU directive.
- No formal requirement with regard to level and area of education is put forward by the directive.

67.3.2 Authorities and Enforcement

For members of the European Union, there is a requirement to register and accredit or license all establishments involved in the procurement, processing, or storing of human cells and tissue for pharmaceutical or therapeutic purposes. These requirements are specified in the EU Commission Directives and their annexes, which thus provide a regulatory framework. Each country then applies this framework onto its own institutions and confers the authority to license and accredit and to perform inspections of tissue establishments. The designated national body may decide to use existing industry standards (e.g., the ISO 9000 series) as components of the regulatory system and may, thus, outsource part of the regulatory oversight. However, the ultimate responsibility rests with the designated national body.

The scope of the directives is very broad, and, as a consequence, the technical solutions or standards required for compliance are not specified in detail for every area, but guidance is found in the annexes. In some areas, the annexes are very specific, but most detailed requirements are instead specified by the designated national body. A consultative forum, constituted by representatives from all the specialties, may be used to support this body in determining the specific technical requirements. The European Society of Human Reproduction and Embryology (ESHRE) has also been active in this respect. Each tissue establishment must have a responsible person, who must be suitably qualified and who is responsible to the authority for ensuring that the establishment is compliant with the directives.

After verifying that a tissue establishment complies with the directives, the authority shall issue a license for the tissue establishment for specified activities. In order for the tissue establishment to retain the license, it must be subject to inspections of the laboratory and clinic, to be undertaken at regular intervals (not longer than 2 years). Annual reports of the clinical and laboratory activities must be submitted to the authority. The inspections should evaluate all processes and documentation pertaining to the directives, and the authority must be granted access to premises and documents as required. The findings of the inspections shall be available to the commission and to other member countries.

Adverse events must be reported, and the authority may decide to inspect the establishment after such events. Also, substantial changes in operation of the clinic/lab must be reported and approved as well as any change of key personnel at the clinic/lab. If a tissue establishment fails to comply with the directive, its license may be withdrawn by the authority. However, if it can be shown that compliance on a particular technical point is detrimental to the tissue, the compliance requirement may be waived for this particular situation.

The European Commission has published an operations manual for the competent authorities regarding inspection of the tissue establishments [5]. This manual describes, for example, the requirements for the inspectors, who should have a relevant university education and previous work experience in this or related fields.

In most European countries, the authorities verifying the implementation of EUTCDs and their performance are pharmaceutical specialists. Specialists from clinical embryology are rarely a part of inspection authority. The inspection process includes checking of all segments described in EUTCD and should be performed minimally every 2 years. Every ART center should designate a responsible person ensuring that human tissues and cells intended for human applications are handled, stored, and distributed in accordance with EUTCD and with the laws in force in the member state. This person must inform the authority in cases of adverse events and reactions that could harm the biological material and could have or have had health consequences for people involved in the treatment with cells or tissues. Although the responsible person for EUTCD is usually the medical doctor, the head of the IVF laboratory is an indispensable participator in the inspection because most of the EUTCD details in the field of ART are related to the work of the IVF laboratory.

The inspectors have to prepare the report about all evaluations and verifications and list the inconsistencies observed during verification. The inconsistencies are classified as critical, bigger, and smaller recommendations. For critical inconsistencies, a very limited time is given to the tissue establishment for their elimination, and an extra inspector's visit can be expected in such cases. The tissue establishment has to provide a timetable and a list of future activities to the authority for the elimination of inconsistencies. Their elimination and prevention or corrective actions are usually an embryologist's obligation.

With the introduction of EUTCD, the inspections, and the legislative obligations to report huge amount of data from everyday clinical and laboratory work to different authorities and registers, reproductive medicine became one of the most regulated and controlled branches of medicine.

67.3.3 Andrology Laboratories

Andrology laboratories that perform semen analyses only are usually under the rules and standards available for general medical diagnostic laboratories. Although the legislation for andrology laboratories is not as strict as for therapeutic laboratories, they have to follow specific standards, mostly provided by the WHO. The last edition of WHO laboratory manual for the examination and processing of human semen from 2010 has a larger part of the manual devoted to the standardization of semen analyses with the aim to minimize the intra- and interobserver variability in sperm assessments. Many international institutions provide their assistance in verification of individual objectivity and accuracy in semen analyses by comparing the individual measurements of semen samples with the measurements of the same samples in reference laboratories.

Andrology laboratories perform a kind of cytologic analysis. That is why they are sometimes under the patronate of cytologists/pathologists as it is the case in the UK.

The andrology laboratories which perform sperm preparation techniques for ART have to work under the same conditions as IVF laboratories, fulfilling all requirements described in EUTCDs.

67.4 Facilities

67.4.1 General Aspects

The high demands on air quality in terms of particle counts and presence of microorganisms made by the EU directive were one of the areas that caused the most concern among ART professionals. Performing IVF under clean room conditions (class A) is certainly very difficult to achieve in practice and may be detrimental to oocytes and embryos because of cooling effects of the high rates of airflow required to maintain such conditions [6]. The final version of the EU directive which, with annexes 1.A–1.D, is in force allows for exceptions under certain circumstances, namely, when a validated microbial inactivation or validated terminal sterilization process is applied, where it is demonstrated that exposure in a grade A environment has a detrimental effect on the tissue or cell concerned, where it is demonstrated that the mode and route of application of the tissue or cell to the recipient imply a significantly lower risk of transmitting bacterial or fungal infection to the recipient than with cell and tissue transplantation, or where it is not technically possible to carry out the required process in a grade A environment. European countries have, in general, adopted exceptions 2–4 for ART, and in these cases, a lower standard of air quality may be applied: class D, according to EU GMP criteria, Annex 1. Particle counts and microbial counts are specified with a maximum allowed level of 3,520,000 particles larger than 0.5 μm, 29,000 particles larger than 5 μm, and 100 colony-forming units (CFUs) collected on sedimentation plates under a period of 4 h. For further details, see relevant GMP criteria.

This standard of air quality specifies particle counts of laboratories "at rest" only and applies to all facilities where oocytes, embryos, and/or sperm are procured, processed, stored, and used for assisted reproduction. Although this level is not very difficult to reach in a room with minimum activity when measurement takes place, the directive also dictates that each ART clinic/lab must show that the conditions under which the operations are performed are sufficient in terms of the above parameters. It must be demonstrated and documented that the chosen environment achieves the quality and safety required taking into account the intended purpose, mode of application, and immune status of the recipient—that is, the burden of proof lies on the clinic/lab to show that it is operating under safe conditions. Documented hygiene routines must be in place, and the planning of the facilities must be adapted to the procedures.

Other aspects of air quality such as the presence or maximum allowed levels of volatile organic compounds (VOCs) are not specified by the directive although this may well be significant in the context of ART. This can however be regulated at the national level and should be considered in the quality system in each ART clinic. General aspects of laboratory standard such as restricted access, entrance log, definition of levels of access for personnel, alarm for unauthorized access, alarms for low O_2 in liquid N_2 storage areas, and alarms for low levels in CO_2 tanks and liquid N_2 storage tanks are not specified in the EU directive but are inferred and should be considered in the quality system of the ART laboratory (see discussion on equipment below).

The standard of facilities and air quality specified in the EU directive is at a reasonably attainable level for a modern clinical laboratory. The exceptions mentioned above for ART cannot however be considered to apply for more advanced forms of clinical applications such as processing and storage of ovarian biopsies for later retransplantation to the patient. Laboratories offering such treatments may need to consider applying higher laboratory standards. In ◘ Table 67.1, the minimum obligatory standards for laboratory quality for the ART clinic and laboratory are specified. Note that no specifications regarding, for example, volatile organic compounds are given, even though data is available regarding the importance of carefully controlling these substances (for a review, see [7]).

◘ **Table 67.1** Laboratory standard for ART

	Obligatory specifications	Parameters for each clinic/lab to specify
Particle counts	3,520,000/ m^3 > 0.5 μm	
	29,000/ m^3 > 5 μm	
Microorganisms	100 CFU	
Access		Restricted
Access log		To be considered
Alarm for unauthorized access		To be considered
Changing facilities		To be considered
Alarm for low O_2		To be considered
Number of air changes per unit of time and positive pressure		To be considered
Direct sunlight		Avoided
VOCs		To be considered
Hygienic routines		To be considered

Minimum obligatory standards for the ART laboratory according to EU regulations

67.4.2 ART Facilities

ART clinics must have designated and suitable facilities for their operations.

Rooms for oocyte pickup and embryo transfer, laboratory facilities for culture, processing, storage, cryopreservation, etc., must fulfill at least class D (ISO class 8) air quality, although local higher standards may be applied. In practice, this is in most cases taken to mean:

- Restricted access
- Changing room be close to the laboratory/operation room
- HEPA-filtrated ventilation
- Positive pressure from lab/operation to surroundings
- Conditions be specified with regard to the above points:
  - Particle count and microbial count
  - Rules for access be documented
  - Hygienic standards be established
  - Laminar airflow hoods be used for cell culture work

67.5 Methods and Quality

67.5.1 Equipment and Materials

One purpose of the tissue directive is to minimize the risk of adverse effects on the tissue by handling, processing, or storing. Therefore, it is a basic requirement that all equipment and materials are fit for purpose. If possible, all materials and equipment used must conform to the requirements of Council Directive 93/42/EEC of June 14, 1993, concerning medical devices and Directive 98/79/EC of the European Parliament and of the Council. This should preferably be evidenced by the equipment and materials being CE marked.

The tissue establishment shall identify equipment and materials that are critical to the safety and the function of the tissues. The suppliers of these materials and equipment must be evaluated and assessed regularly for compliance by the tissue establishment. It is furthermore required to validate the function of the equipment and materials and document the results of the validation. Also, the establishment must have documented plan for maintenance and replacement. There must be documentary evidence of maintenance occurring and that the equipment remains in conformity with the directives. Measuring equipment should be calibrated against a traceable standard, where available.

In order to ensure that the equipment and materials function as required, there must be instructions for their use and documentary evidence that staff have the competence to use the equipment and materials correctly. The tissue establishment must document which individual (critical) equipment and which batch of materials have been used for each sample of tissue. The function of critical equipment must be continuously monitored and all data logged. An alarm system must be fitted, so that corrective action can be taken quickly in response to malfunction. Backup equipment must be available for critical systems.

It is important that the environment is maintained in a way that allows the equipment to function properly. This includes reliability of utilities, such as electricity and gas supplies, as well as control of temperature, humidity, and other physical variables that may affect the function of the equipment. Also, storage facilities for materials must conform to the requirements of the materials so that their properties do not change during storage. For example, culture media should be stored under the conditions specified by the manufacturer. There must be documentary evidence of measures to control the environment.

67.5.2 Traceability and Documentation

Tissue specimens must be appropriately labeled, so they are identifiable with regard to what they are and from whom they were taken. The fate of the specimens must also be recorded. When the tissue is collected, moved, used, or discarded, this must be recorded also, and consent for these actions must be documented and archived. In order to ensure that the information is accurate, there must be regular audits of the tissue specimens.

It is, moreover, a requirement that the methods of handling, processing, and storage of the tissues are documented in detail and retrievable when the tissue is to be used. For example, the documentation of embryo freezing must contain a detailed description of the freezing protocol as well as information on which medium was used. Thus, it is not sufficient to just state that "embryos were cryopreserved by slow freezing with propanediol." The exact protocol used at that time must be available, as must information on the specific type of medium used. This information ensures that appropriate methodology and media are used for thawing. Furthermore, if an adverse or otherwise significant outcome has been associated with a certain protocol, it is important to identify all tissues exposed to that protocol in order to inform affected practitioners and tissue recipients of these events.

All materials that come in contact with the tissues must be identifiable by product description and lot number. Again, if an adverse or otherwise significant outcome has been associated with a certain product or product lot, it is important to identify all tissues exposed to that protocol in order to inform involved practitioners and tissue recipients of these events. This is, of course, particularly relevant when biologically derived materials are used and there is a risk of disease transmission through these materials, for example, gonadotropins or human serum albumin for culture media. Thus, tissue culture plastics, liquid handling consumables, ET catheters, aspiration needles, culture and handling media, etc., must all be traceable.

It is a duty of the tissue establishment to ascertain that, when receiving/dispatching tissues, the dispatching/receiving establishments adhere to the standards set out in this directive. Special regulations apply for the export and import of tissues to and from other countries (see below).

As mentioned above, the tissue establishment must have a documented system for quality management. This system should contain all guiding documents, that is, instruction how to perform a procedure. The system should also contain all reporting documents, that is, forms. All documents should be reviewed regularly and reauthorized. This is to ensure that the documents are up to date and that there are documents covering all procedures and also to allow culling of documents no longer required. It is of great importance that only the latest version of any document is in use and therefore distribution must be controlled. Old versions of a document must be recalled when a new version is issued, and the use of nonauthorized documents should be discouraged. For reasons given above, old versions of documents must be archived. Document control is demanding, but there are software systems to assist the quality manager in this regard. Standards for quality management systems, for example, the ISO 9000 series, provide good guidance for document management.

For all documents issued, it must be clear who has authored it and when. Reporting documents must state who did what, when, and with what authority (position in tissue establishment, e.g., embryologist) and then be signed. All staff involved in a procedure should be identified. Records may not be changed without proper authority, and there must be an indelible log of all changes to records. Consent to procedures by patients and donors involved must be documented, and national standards apply for the process of consent taking. Raw data forms should be stored for 10 years. Information on donors and patient notes must be kept for a minimum of 30 years after the tissue was used.

Documents must be legible not only at the time they were created but also for the duration of the archiving period. Handwritten notes rarely meet this requirement. There should be mechanisms in place to ensure that the information contained is complete and accurate. This could be achieved by double witnessing and audits.

Access to the documents should be tightly controlled to prevent unauthorized changes and, above all, to prevent unauthorized access to confidential client information. This can be implemented by a combination of physical and software-based solutions. Regulatory authorities must be given access to documentation and records for inspection and control.

If the clinic ceases operations, all documents must be transferred to another tissue establishment. There must be a documented plan for this contingency.

67.5.3 Quality Management

A documented quality control system must be established in every ART laboratory. Establishing such a system is a complex task, and most laboratories find it useful to rely on some of the published standards, which define such systems and describe their function. The most commonly used of these standards are as follows:

- ISO 9001, commonly called certification standard, since competence testing of the procedures in question is not implicit in it. This standard is commonly used in many establishments and in the industry and has a strong focus on continuous quality improvement.
- ISO 15189, commonly used for establishing a high level of competence in medical laboratories.
- GLP/GMP standards, which are often used in the pharmaceutical industry.

Details on the differences between these standards are beyond the scope of this chapter and are not discussed further here.

The ART laboratory does not have an isolated existence. Some laboratories may operate in a highly independent manner, but most are integrated into the ART clinic. Therefore, irrespective of the standard applied in the ART laboratory, the clinic may decide to establish a corresponding quality control system by utilizing the ISO 9001 standard.

The first step taken toward establishing a quality control system is usually to systematically document all procedures (standard operating procedures, SOPS). Computerized solutions may be of great assistance in this respect. Other aspects of quality control are, for example, registering and process of nonconformities, internal and external revisions, and continuous improvements in processes and procedures. In Table 67.2, the requirements of the EU directive in this respect are specified and simplified as a comparison to the ISO 9001 standard.

67.5.4 Methodology

The methodology used for collecting, transporting, processing, and storing tissues must protect the critical characteristics of the tissues and cells. For gametes and embryos, this means preserving the ability to participate in fertilization and retaining the ability to develop normally. All procedures used

Table 67.2 Simplified comparison between the EU directive and the ISO 9001 quality management standard

	Directive 2004/23/EC	ISO 9001
Document all procedures and SOPs	X	X
Document control	X	X
Document checklists	X	X
Training manuals	X	X
Guidelines	X	X
Nonconformities	X	X
Internal/external audits	X	X
Process analysis		X

must be documented and validated prior to being used with a view to ensuring that this requirement is met. This validation can be made in-house or be based on published good-quality scientific studies. All procedures carried out at the tissue establishment should be monitored in order to ascertain that the outcomes meet acceptable standards and that performance is consistent, and regular audits must be performed. When possible, inter-establishment validations should be conducted. The validation procedures should be documented, as should all outcomes from the validations and monitoring activities. Any changes to any critical procedure must be followed by a revalidation of the procedure. A more detailed guidance on methodology for ART clinics can be found in good practice guidelines issued by international bodies like ESHRE. Procedures should be carried out only by staff with verified and documented competence. Staff should be regularly assessed for competence in performing procedures.

67.5.5 Coding

A single European coding system is now established for all tissue establishments [8]. Exception is made for partner donation, and a possibility for exception is also allowed for member states to give for tissues and cells other than reproductive cells for partner donation, when these are used and remain within the same center. Information on the coding system is available at the EU website (► https://webgate.ec.europa.eu/eucoding/), and the system came into force in April 2017. In addition to the code for the tissue establishment, an international code such as ISBT 128 for the tissue/cells in question may be used. The ISBT 128 system is currently used for blood, tissue, and organ identification (► http://iccbba.org/).

The implementation of a single European coding system will have major effects for ART clinics in years to come. Some form of bar-coded identification system (such as ISBT 128) will almost certainly be implemented, meaning further standardization of labeling and laboratory processes as well as increased demands on computerized labeling and documentation systems. Even though such labeling systems may not be obligatory for ART with partner donation, it will be difficult for any ART laboratory to avoid implementing them in the long run. This is partly not only because the old methods of labeling will soon be considered obsolete when the larger clinics implement the new systems but also because any use of donated gametes will demand laboratory competence in handling such systems even though the clinic in question may not have a donor sperm bank since many ART laboratories handle oocyte donation. It is relevant to note that most medical laboratories, such as blood banks and analytical laboratories, have such computerized systems in place today, and in this respect, the typical ART laboratory may be considered some years behind current development.

67.5.6 Cryostorage of Gametes, Embryos, and Gonadal Tissue

Cryostorage of cells and tissues is an important aspect of the EU directive as well as for ART laboratories. Maximum storage times must be specified according to the directive and an inventory system established. Procedures regarding all aspects of the storage system must be documented, and all laboratory tests must be completed before release of products.

Regarding details on storage, such as selection of a suitable carrier system for sperm, oocytes, or embryos, as well as selection of cryotanks, this is up to each laboratory to decide. Risk of cross-contamination must however be eliminated. Therefore, gas-phase storage and use of sealed containers have been extensively discussed in the context of ART as well as the relative risks associated with controlled-rate freezing vs. vitrification [9].

The current interpretation of the situation is that gas-phase storage in assisted reproduction is not considered mandatory for oocytes and embryos since this may compromise the cells because of the small volumes involved. Gas-phase storage for sperm samples may be recommended but is not considered mandatory since secure sealed containers are available. As a general rule, sealed containers are highly recommended wherever possible to ensure a controlled environment for the gametes/embryos irrespective of cryopreservation method. Audit of storage tanks and records must be performed each year, whereas time limits for storage vary according to local legislation.

67.5.7 Laboratory Tests

Tissue donation involves a risk of disease transmission, and for that reason, donors must be tested for certain communicable diseases. The situation in standard assisted reproduction, involving a couple in a stable relationship, is special in that the participating individuals are already exposed to this risk, so screening will not prevent horizontal transmission. However, vertical transmission to the offspring may be prevented by screening, and in cases with donation from a third party or gestational surrogacy, screening is essential. Genetic screening is also performed in certain risk groups that may be carriers or at risk of genetic conditions.

For standard assisted reproduction, couples undergoing treatment must be screened for viral infection, such as HIV 1 and 2, HTLV I & II and hepatitis B and C, as well as syphilis. Sperm donor should be screened for *Chlamydia* infection. Screening for rubella immunity and vaccination reduces the risk to the fetus of becoming infected and developing the congenital rubella syndrome, a common consequence of which is deafness. Depending on the local disease spectrum, the designated national body may determine that additional screening is needed. The interval for retesting in ART is currently implemented at 2 years in most, if not all, EU countries.

Donors need to be screened prior to donation, the donated tissues then need to be quarantined for 6 months, and the donors are retested before release. However, if the initial screening assays involve nucleic acid amplification techniques of the samples, quarantine and repeat screening are not required according to the EU directive. The laboratory tests must be performed by a competent laboratory that has been authorized by the designated national body. This requirement is often interpreted as a requirement for ISO accreditation of the assays in an ISO-certified laboratory.

For partner donation, a positive test result does not necessarily exclude treatment. Here, the situation is determined by the national authorities. However, in all other cases, a positive infection test results exclude the donor.

67.5.8 Import and Export

According to the EUTC Directive, the European Union is considered one area with free import and export between countries since the same regulations and minimum requirements apply within the EU. Restrictions apply however concerning import and export to and from countries outside the EU. Member states must take necessary measures to ensure that imports and exports of tissues and cells from and to third countries are undertaken by tissue establishments accredited, designated, authorized, or licensed for the purpose of those activities and that imported tissues and cells can be traced from the donor to the recipient and vice versa. Several member states have established registers of authorized tissue establishments in this respect.

67.5.9 Reporting

Volume of activities at the ART laboratory must be reported in some detail to the local authorities. This includes the number of oocytes and sperm samples collected, inventory status of cryostored samples, and number of samples destroyed. Local forms for this purpose may be established. In addition to this, all adverse events and serious adverse events in connection with ART activities must be reported to the local regulatory authorities. Exact definitions of what constitutes an adverse event or serious adverse event are not available in detail; however, some examples may be mentioned. All cases of identity mix-up are considered serious adverse events as well as any cases of transmission of infectious disease in this context. Inventory irregularities in cryostorage should be considered an adverse event, whereas reduced cell survival after thawing, which is difficult to avoid in many cases, should not be considered such an event. Accidental destruction of cells during processing must be considered an adverse event, whereas failed fertilization in many cases cannot be considered such an event. Ovarian hyperstimulation syndrome (OHSS) is usually not considered an adverse event in the context of the tissues and cells directive, whereas local health authorities may require incidence of OHSS to be reported. For details regarding these aspects, local authorities must be consulted.

67.6 Legal Aspects and Local Legislation

Although implementation of the EU directive on tissues and cells calls for coordination of regulatory demands on ART in Europe, local legislation varies considerably. Public financing is one such area as most European countries offer some public funding of treatment and a few offer full coverage for at least a number of treatments. Usually this funding is contingent on age limits and/or medical indications for treatment. Usage of ART varies greatly among European countries, where affordability is a driver of usage [10]. Other areas of variation include availability of treatment for single women or lesbian couples with donated sperm, donor anonymity, time limits for cryostorage of oocytes and embryos, and the use of genetic diagnosis and genetic screening for treatment purposes and embryo research – to name a few examples. For further information on the variation of legislation within Europe, see also Berg Brigham et al. [11].

67.7 Clinical Embryology in the EU

Clinical embryology is a young branch of laboratory medicine which covers a part of therapeutic procedures in the treatment of infertility. Staff working in ART laboratories closely collaborate with gynecologists during most steps of the treatment. Together, they represent the core of the reproductive medicine, young gynecological subspecialty.

Clinical embryology started with its development in research and improvised laboratories. Today, all ART methods must be performed in high-tech laboratories with sophisticated equipment ensuring the optimal and constant physicochemical conditions, appropriate for the beginning of human life, development of human embryos, and their culture in vitro until the blastocyst stage. With the introduction of micromanipulation techniques for mechanical fusion of gametes and for biopsies on embryos, it became evident that the personnel from IVF laboratories require special education and long-term training.

According to the estimation of ESHRE, around 1400 IVF clinics existed in Europe in 2012/2013. Around 70% of them were private and 30% were public. Half of all IVF clinics were ISO certified. In 2012/2013, there were around 7000 laboratory workers who performed 540,000 fresh IVF cycles and 160,000 frozen embryo replacements [12].

In around 80% of European countries, the IVF laboratories are operated by embryologists/scientists with MSc or PhD. Physicians are rarely the managers of IVF laboratories with the exception of France, Poland, Romania, and Russia where they predominate. The analysis of the educational structure of remaining personnel working in IVF laboratories showed that in the Northern and Western Europe, the prevalent profile was that of laboratory technicians without

or with BSc. Personnel with university education in natural sciences and MSc or PhD were in minority. The situation was opposite in the Southern and Eastern countries.

The title clinical embryologist had various interpretations within and among the European countries, but it was an officially recognized occupation in national health-care systems in only three countries. In other countries, the personnel had to be registered under another official occupation. Among all European countries, only the UK had clearly defined requirements for accessing the title clinical embryologist.

The survey showed that only 40% of the countries had at least some form of an official document that vaguely defined the requirements for managing IVF laboratories. In the remaining countries, no such document existed. Even more worrisome is the fact that organized postgraduate education or training for independent work in IVF laboratories was reported to be mandatory in only four European countries. In other countries, the only way for acquiring practical experience and knowledge was reported to be by transmitting the experiences of more trained to the less trained staff.

For now, there is no agreement on uniforming the European organized training in ART or the specialization of clinical embryology. Only few countries regulate this field on their own, mostly by certification of staff using national rules. The only international examination of theoretical knowledge from clinical embryology, which attracts the highest number of embryologists from all European and also non-European countries, is the ESHRE certification. The ESHRE certificate is not recognized as an official document in most of the countries. However, the certification of knowledge from clinical embryology is one of the minimal standards mentioned in the revised guidelines for IVF laboratories [12].

ESHRE is continuing in improving the embryologist certification program by also introducing the system for the evaluation of continuing professional development (ESHRE-CPD). ESHRE's further activities in improving the professional status of clinical embryology and clinical embryologists will be focused on European institutions, responsible for medical specializations and examinations.

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Review Questions

1. Which of the ART laboratory processes should not be considered to be included in the exception of laboratory standard specified in the EUTC Directive?
2. What is the current interpretation on gas-phase vs. liquid-phase storage for reproductive cells?
3. Which laboratory tests should be performed for patients coming for treatment in the ART clinic?
4. Which of the ART processes are exempted from the unified EU coding system implemented in April 2017?

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Regulation, Licensing, and Accreditation of the ART Laboratory in Brazil

Fabiola C. Bento and Sandro C. Esteves

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Learning Objectives

This chapter aims at informing the reader of the following:

- Current ART regulation in Brazil
- Air quality requirements
- Quality management system (QMS) requirements
- ANDROFERT experience with QMS

68.1 Introduction

According to the International Federation of Fertility Societies (IFFS) latest surveillance report, there are approximately 5353 ART centers around the globe. Only 57.1% of the countries have to follow specific legislation regulating ART activities, while 24.3% have only national society guidelines without any formal legislation, and 18.6% have no regulatory structure in the form of legislation or guidelines [1].

In Brazil, the regulatory agency (ANVISA) issued regulations for reproductive cell and tissue banks, which include assisted reproductive technology (ART) centers, in 2006 [2]. This directive was later reviewed, in 2011, to include more details and explanations not very clear in the first resolution [3] and was again reviewed in 2016 to include specific testing instructions due to the Zika virus outbreak and microcephaly cases [4]. Besides the regulatory agency directive, ART centers must observe ethical standards established by the Medical Council (CFM 2121/2015) [5], a professional organization that doctors and clinics must comply with and whose objective is to guarantee the safety and efficacy of medical procedures and treatments involved in ART.

ART units providing assisted reproduction treatments involving in vitro manipulation of gametes and embryos that operate in Brazil are therefore obliged to comply with specific regulatory requirements, as defined by standards of quality and safety for the donation, obtaining, testing, processing, preservation, storage, and distribution of human tissues and cells (Table 68.1). To operate, ART units must have a license permit issued by the regulatory agency (ANVISA) and must renew it annually.

Table 68.1 Summary of regulatory requirements in Brazil

Requirements	RDC/Anvisa #72, 2016	CFM 2121/2015
Quality management system	X	
Infrastructure and equipment	X	
Air quality control	X	
Written consent for assisted reproduction treatments	X	X
Age limit for female patients		X
Requirements for patient selection and donor selection	X	
Sex selection prohibition		X
Limitation on number of embryos transferred according to age		X
Fetal reduction prohibition		X
Same sex and single parenting allowed		X
Rules for oocyte, semen, and embryo donation and anonymity	X	X
Rules for cryopreservation of oocytes, semen, and embryos	X	X
Preimplantational genetic diagnosis		X
Rules for gestational carriers, who must be family members		X
Rules for discarding embryos	X	X

68.2 Air Quality Requirements

A few regulatory agencies have issued directives that include specific requirements for air quality control in embryology laboratories. Fertility centers holding assisted reproduction technology (ART) units in countries where such regulations are in place should operate in conformance with these directives. The basis for mandating ambient air quality control is to safeguard public health preventing transmission of infectious diseases via transplanted tissues and cells, according to the premises of the precautionary principle. The precautionary principle is used when measures are needed in the face of a possible danger to human health where scientific data do not allow a complete evaluation of the risk.

The Brazilian directive dictates that laboratory air quality for particulates should be at least equivalent to ISO class 5 in the critical areas where tissues or cells are exposed to the environment during processing. One of the following methods is recommended to achieve such conditions: (i) biological safety cabinet class II type A, (ii) unidirectional laminar flow workstation, and (iii) cleanroom at least equivalent to ISO 5.

Laboratory background air, which includes indoor air in areas for carrying out less critical stages, should be pressurized and filtered for particulate matter when biological safety cabins and unidirectional laminar flows rather than cleanrooms are applied. Areas in which oocytes/reproductive tissues/sperm are surgically retrieved should be pressurized as well (Table 68.2) [6].

Table 68.2 Ambient air quality requirements for in vitro fertilization laboratories operating under regulatory directives in Brazil

Particle filtration	At least equivalent to ISO class 5 (NBR/ISO 14644-1) in the critical areas where tissues or cells are exposed to the environment during processing
Microbial contamination	Microbiological monitoring required, specifications not clearly defined
Volatile organic compound filtration	Ventilation systems should be equipped with filters imbedded with activated carbon

Adapted from Esteves and Bento [6]

68.3 Quality Management Requirements

Besides all technical requirements for operation, the Brazilian regulatory agency requires the establishment of a documented quality management system (QMS), which should include:

1. Elaboration and periodic review of standard operating procedures (SOPs)
2. Periodic personnel training
3. Periodic internal audits, to verify compliance with technical regulations
4. Procedures for detection, registration, correction, and prevention of errors and nonconformities
5. Compliance with biosafety regulations
6. A system to evaluate and control equipment and materials used

Many other demands also related to a quality management system are included in the regulation, such as:

1. Having a technical manual with:
    1. Administrative and technical organization
    2. Qualification and responsibilities of all professionals involved
    3. Conduct in case of nonconformities
    4. Biosafety norms
    5. Annual review
2. Having proper sample identification
3. Keeping at least two copies of all data (in other words, a backup system) and guaranteeing that data cannot be altered at any time
4. Having a maintenance plan for all equipment

68.4 ANDROFERT Quality Management System Experience

After making an extensive research, we at ANDROFERT decided to follow the International Organization for Standardization (ISO) 9001 quality management model [7], first because it included all basic aspects of the Brazilian directive and second because the ISO is recognized worldwide as the most complete quality management model.

We started by creating our "nonconformity and corrective action registration procedure" and allowing anyone from any area to record nonconformities, even if the nonconformity was not from this person's area. Specific training was given, and even though registering nonconformities was rather easy, responding to nonconformities was the greatest problem. Most people think only in immediate actions to solve whatever problem happens or think of solutions that do not address what caused the nonconformity in the first place. Analyzing the cause of the nonconformity was our main challenge but was of crucial importance to consolidate the idea of continuous improvement.

At the same time, we started a lengthy training program, focused on ethics, moral values, organizational values, etc., to emphasize the importance and responsibility of each one in the process of offering a quality service. The creation of the concept of "teamwork" was our primary goal.

Another concept that had to be established and fully accepted was that of "continuous improvement." It is not easy to be criticized if it is seen negatively. Therefore, it was important for all staff to learn to accept this criticism and mainly to see it positively, as a means to achieve our ultimate goal, which is offering a high-quality service. To avoid competition among colleagues and make people focus on our common goal, nonconformities were registered in the name of the area in which it had occurred rather than in the name of the person involved. In other words, we avoided the direct criticism of any individual team member and worked on the idea of teamwork directly. They had to respond to the nonconformity as a team. This teamwork led them to develop a closer bond with each other and also taught them that they depended on each other to have a good performance. Performance was not seen individually; therefore, it required some time for people to adapt to that, share responsibilities, distribute tasks, and really work together instead of simply sharing the same workspace. A deep change occurred.

As for our SOPs, in our laboratories, all procedures were already described and reviewed periodically. Besides that, we already controlled all equipment and had a maintenance plan in place. The maintenance periodicity was established and differentiated according to the use and importance of the equipment.

Regarding our clinical activities, almost all procedures were described too. We already had a description of most procedures performed by nurses and doctors. All those not described were then formalized and documented to guarantee uniformity.

Our administrative procedures had no descriptions at all. To start with, we decided to create different flowcharts showing the moment a patient calls to schedule appointments and tests, the moment a patient comes into our center for any procedure, and so on. From those, we were able to determine the activities performed by our administrative staff and

describe them. We identified and formalized all procedures performed within the center, trained all staff, and therefore were able to monitor whether or not they were being done correctly.

While developing and describing SOPs, we established our mission statement and came up with our quality policy, and later with our policy objectives, which determine what aspects of our quality system are evaluated and monitored.

As for our laboratory performance, we determined the indicators that were going to be monitored, such as fertilization rates and embryo development. We also determined how often they were going to be analyzed. This "quality control" was already in place before the establishment of the ISO system. We just had to improve it, setting meetings to analyze data in a more systematic way, recording all actions decided during these meetings, and adjusting parameters to better comply with worldwide performances and also to continuously improve our own.

With the establishment of a paper-free laboratory and development of our own electronic information system, this data can be monitored daily. Every patient's data that is added is automatically used in calculating indicators, so today we have results in real time, without having to calculate any rate or making any data survey.

68.5 Conclusion

Assisted reproductive technology units providing assisted reproduction treatments involving in vitro manipulation of gametes and embryos that operate in Brazil are obliged to comply with specific regulatory requirements for the donation, obtaining, testing, processing, preservation, storage, and distribution of human tissues and cells. To operate, ART units must have a license permit issued by the regulatory agency that must be renewed annually.

ART units operate under a regulatory directive that requires cleanroom standards to reproductive laboratories. The regulatory directive aims to safeguard public health in line with the precautionary principle, thus preventing transmission of infectious diseases via transplanted tissues and cells, but it does not specifically address how periodic testing and validation of these areas should be carried out.

ART units in Brazil must also have a quality management system; however, the regulation does not require a certification. Instead, it determines all items that should be included in the system implemented.

Review Questions

1. Do all countries have specific legislation regulating ART activities?
2. Why is air quality control mandatory in some countries? What is it based on?
3. What should be included in the QMS required from ART centers in Brazil?

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Troubleshooting in IVF Laboratories

James Stanger

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Learning Objectives

- To appreciate the complexity of the IVF system
- To identify the sources of risk and variability
- To create tools to manage the IVF system
- To develop and manage key performance indicators (KPIs)
- To develop a sound approach using KPI history to troubleshoot the performance of an IVF clinic

69.1 Complexity of IVF Systems

IVF is something that just should not happen. The key element in all life systems is reproduction, and, in mammals, it is both highly managed and through survival of the fittest, driven to succeed. Enormous evolutionary effort has been devoted to ensure reproduction occurs from delayed puberty and subtle endocrine controls, from complex systems for gamete production and maturation and from implantation and parturition. Reproduction is unimaginably complex. Social and environmental interactions add even more complexity. Yet within a very short period of time since IVF came into being, it has turned into a universal and transportable activity and industry. During its development, IVF researches have dismantled some control systems such as follicle development and ovulation, sperm selection and natural selection of gametes and replaced them with other control mechanisms in some way more complex than nature. GnRH antagonists, intracytoplasmic sperm injection (ICSI) and preimplantation genetic diagnosis are classic examples of the new norms.

IVF activity is more than just biology; in fact, biology of reproduction is almost a side issue. IVF clinics by and large do not attempt to achieve pregnancy in fertile healthy couples; rather it seeks to achieve pregnancy where nature has failed. Apart from social activities such as same sex or single women's conceptions, IVF is largely about trying to make the impossible possible. In many ways, IVF has been remarkably successful with pregnancy rates close to or above those seen following the honeymoon period for fertile couples (OK, maybe recent social changes have dented this somewhat but pre-contraception and universal marriage alliances saw conception rates of more than 20% per month and 80% in the first year). Rates of more than 50% per transfer are increasingly the normal expectations in the most fertile of an infertile population (under 35 years of female age and the first 2 treatment transfer cycles).

This has several consequences. Firstly, it puts pressure on all clinics to try and maintain such high goals as more and more low-probability clients request treatment. Secondly, it may produce a generation of scientists that may never have experienced poor outcomes and be poorly prepared to deal with them, and, thirdly, much of this success has been due to applying increasingly complex technology such as PGD, thereby increasing the possible likelihood of system failure.

69.2 Defining Expectations

The complexity of IVF systems may start with the most logical ones such as the laboratory process, but more importantly it is overlaid by individual clinic dynamic ones such as patient mix, staff mix (especially clinical activities since clinical personnel continue to this day to see themselves above variation), staff competency, treatment mixes, technology mixes (CSI, PGD, cryopreservation) and activity loads. The responsibility to manage this usually falls to a quality manager or if not designated and then usually to a scientific staff member. If the net activity of all these sources of variation is carefully managed, they approach a productivity that is relatively stable, as occurs in established larger (but not too large) clinics. In such periods, there may be little need to troubleshoot. The issue is when to troubleshoot and when not to. Changing systems because a medical director says things are declining is not necessarily desirable since when any change is instituted, then there must follow a period of review. In my view it is better to develop a system of management tools to provide clues if the system is failing or if the system is changing, is responding to changes and innovation or is just moving within its natural limits. These two perspectives (failing and changing) may both produce a decrease in productivity, but troubleshooting them is quite different.

Just because the pregnancy rate in a recent period is lower than previously or in comparison to other clinics (say in an annual accreditation review) does not mean the laboratory is the cause. Impetuous changes to systems may result in more variation than desirable.

Having made this statement, one is ready to concede that IVF systems are very complex, and lack of focus can easily result in significant decreases in productivity at a biological level, but if the system is stable and well managed and appropriately supervised, then biological-based failure (i.e. gamete or embryo loss of viability) is rare. In approaching troubleshooting in an IVF clinic, one should first establish one's own productivity. This can be measured in a myriad of ways, and the quality manager should utilise as many as possible to know if change is happening or not. More importantly, each clinic should recognise that the clinical and technological process is always undergoing change and needs to develop sets of data, based upon previous performances against which current practice is measured for evidence of problems, and these reference data sets are routinely reassessed and recalculated to provide a realistic tool for comparison, in other words a robust set of key performance indicators (KPIs).

69.3 IVF Clinics Are More Than the Laboratory

At the very basic level, every embryo will develop in vitro with some degree of trauma not experienced in vivo (except where in vivo environments are toxic or misaligned). In fact there is no real information on how a human embryo devel-

ops in vivo, so the above statement is purely conceptual. Experimental work with mammalian and specifically with human embryos has suggested embryos are resourceful in using whatever environment agents are available to develop, and it is presumed that many of these biochemical routes would unlikely ever be used in vivo. Other data suggest that where the environment is compromised, the embryo will commit more resources (energy reserves) in an effort to counter the issues, an action that may reduce their developmental capacity. A good example of this is pH [1].

In terms of troubleshooting IVF labs, the starting point is to appreciate that all oocytes (and presumably sperm) and preimplantation embryos are compromised by the very nature of IVF, and the role of the IVF lab is to minimise the degree of stress, and it is wrong to believe the culture environment is great. It is also worth remembering that the vast majority of embryos will never have the capacity to implant or result in a term delivery and a live baby. The simple fact of the matter is that while great strides have been made in ovarian stimulation and control, the vast majority of follicles do not produce a viable (baby) oocyte. From variation in maturity, size, vascularisation, etc., the complex process of oocyte development remains incomplete or compromised at the time of ovulation induction and oocyte recovery. About 13% of all oocytes may result in a live birth in any cohort, so IVF is about sorting out the "good" from the "less good" oocytes/embryos and that any tool that seeks to measure performance needs to keep in mind that the vast majority of oocytes/embryos will fail [4].

It is against this background that any trauma (loss of quality in the environment) will impact most on the poorer quality gametes and embryos. If one thinks back to the very earliest days of IVF when significant levels of contamination and suboptimal environmental conditions existed, some very healthy embryos still managed to survive and when transferred result in healthy babies and children. Granted not many but the point is that the best embryos will by and large tolerate considerable discomfort and maintain their viability. Even when for a while, embryo fragmentation was considered to be a normal state for human embryos (in the mid-1980s), the pregnancy rates were still about 10–15%.

One further point about trauma is that it starts with a preovulatory oocyte. Unlike cleaving embryos, oocytes are exceptionally vulnerable cells. Again using pH as an example, the mature oocyte has virtually no capacity to self-regulate their internal pH. This capacity increasingly develops after fertilisation and with cleavage such that only after compaction does the embryos really start to control its own internal pH [2]. So any troubleshooting should always start with the oocyte and move forward rather than the final embryo stages and move backward.

The sources of "trauma" would encompass inappropriate:

- Salt or energy concentrations
- Protein and environmental elements such as antioxidants
- Toxic oil
- Culture dishes
- Gas mixture
- Incubator temperature or CO2 concentrations
- Warming stages and other handling environments
- Needles, catheters and other handling tools
- Environmental issues (perfumes, deodorants, renovations, etc.)
- Staff and personnel

The thing with all these sources of trauma is that it can all be easily managed. Each source of stress should be defined and managed. Each clinic may well have a variable list of potential sources of trauma and as part of their quality management programme institute procedures to monitor and minimise variation in them. There may well be many more that a scientific director or quality manager could identify and outline a strategy to monitor. The point is that these are not dynamic but relatively stable and should not change without a conscious and approved decision by the senior team. I will get to staff later, but the golden rule is that all staff, via the quality manual, do not change them.

Each potential source of trauma should have a set of rules and periodic monitoring and recording that are routinely reviewed and certified to meet expectation. As part of a laboratory audit process, the senior quality manager should be able to confirm to the senior clinical management and to auditors and accreditation inspectors that each risk factor is identified, has a monitoring protocol attached to it, is monitored and recorded as per protocol and is reviewed as per protocol. There is an enormous effort (often boring and time-consuming) discharged on a daily basis to ensure these risks remain under control. But the point of all these is to recognise that the potential for all oocytes, sperm and embryos can never improve under IVF culture conditions, only deteriorate. Importantly, when a downturn in productivity occurs, often when a clinician remarks that there have been few pregnancies of late, the quality manager should be able to affirm and prove that the laboratory key parameters are under control.

Let's quickly run through the trauma risks listed above to illustrate how a quality manager may be able to affirm each risk is under control. Many of the views are personal views developed over 30 years on managing IVF laboratories, and it may well be that other scientists may have different views.

69.3.1 Salt or Energy Concentrations

When IVF was first developed, most researchers used media commonly available to routine tissue culture laboratories such as Ham's F10 or Earle's balanced salt solutions. As human IVF developed, so did the realisation that human embryos were not the same as cell lines developed of years to grow in culture and formulations based upon guesstimated tubal fluid concentrations evolved, and their descendants now dominate the IVF industry. They have by and large been

spectacularly successful and formed one of the first quantum leaps in productivity. No clinic can validly prepare their own media these days purely due to cost and the requirements for quality control (QC) and certification. This means that each clinic needs to make a decision which media to use. Often the decision is based on cost or supply issues rather than on whether they are best for embryos. The market place for media is huge, and selection of media is and will remain problematic. Who now knows what is best – I certainly don't and I guess no one else does. There has been a conflict in recent years on whether media should be sequential to reflect the changing needs and environments the embryo encounters before implantation or can a standardised universal media achieve the same result. Do media need to be changed every 2 days or can it provide a stable environment for the complete duration of embryo culture?

The IVF culture system is also undergoing huge changes in the evolution of blastocyst transfers, vitrification, embryo biopsy and analysis and time-lapse monitoring. So the ten golden rules for media are:

(a) Record the media type and batch numbers for every case.
(b) Don't change media or mix and match different media with a good reason. Every time an embryo moves from one type to another, some form of readjustment needs to occur. Every time this happens, more energy is used to adjust and compensate detracting from growth and development.
(c) If you are in the top 50% of clinics according to accreditation documented success rate, don't change.
(d) If you are in the lower 50% of clinics according to accreditation documented success rate, look to change to a media used by what you consider to be a peer clinic.
(e) Use a media that reflects to primary process, e.g. if 80% of transfers are on day 2, why would you change to media that is for day 5 biopsy cycles? If you plan to move from one process to another, make one change only. Staff will struggle with having to use different media in different situations.
(f) Use as few formulations as possible. Many media suppliers have a media formulation for every step of the process that may have a logical basis, but is it necessary? The fewer steps there are the better. Remember that if sperm are held in sperm processing media, then when added to an IVF insemination drop, they will alter the composition.
(g) Remember that with a large cryopreservation programme, embryos put in storage now may not be recovered for several years.
(h) Purchase media in the smallest volume possible that will last for a defined period to allow for *all* media of a set batch number to be replaced at the same time. It is impossible to manage each bottle of media through a QA system if each patient's embryos are exposed to a multiple mix of lot numbers and deliveries. It is my view that it is better to purchase lots of small bottles of the same media and cycle through them in a managed manner than purchasing one large bottle that is repeatedly accessed. This is false economy and guarantees each cohort of embryos will be exposed to a dog's breakfast of new and aged, pristine and exposed media.
(i) Recognise that some wastage is OK, and factor this into your costing rather than use every bottle to the very end. Recognise that every time a bottle of culture media is opened and used, its composition will change slightly and be exposed to contamination. Embryos grown in the first drops from a new bottle to that in the last drop will not be growing in the same media.
(j) From a quality management perspective, it is better to replace media as frequently as possible with new lot numbers rather than purchase large supplies of media to last a long time. In other words, buy media in the shortest delivery time frame possible. On delivery, maybe do a quick sperm survival check and replace all media with the new lot number. Move all the old media to the back of the storage area (refrigerated) and discard prior to the next delivery. The logic is that it is naïve that all media is OK since any one bottle may not meet quality control specifications of the manufacturer and the risk of some form of introduced toxicity is ever present. The best solution to this is to recycle media and their containers to avoid exposure to a large number of clients.

Finally, I have long been a supporter of single-use media containers, whereby a clinic purchased a number of packs of media to service the needs of one client. Each media container is used once and discarded along with unused containers. It will revolutionise quality management, dispense with multiple accessing of containers and allow the manufacturers more control over the supply chain.

69.3.2 Protein and Environmental Elements Such as Antioxidants

Proteins provide both buffering and osmotic buffering functions, a reserve of nutrients and a scavenger function. Early systems using serum were a system with little control. Most culture media supply their own protein source either as integral part of the culture media or as an addition prior to use. Some provide purified serum albumin or recombinant albumin, while others supply albumin as a serum substitute preparation containing albumin plus alpha and beta globulins. Early issues with the production of ammonia breakdown products involving amino acids and protein are largely irrelevant in the current climate.

As for culture media, it is problematic for a scientist to choose any protein source over another, but from a troubleshooting perspective, one may argue the fewer combinations and the shorter exposure times (from the time a bottle is opened), the better. In fact, choosing a media that is delivered with the protein already added makes problem-solving and quality control much easier. So unless there is a clear benefit

in mixing media and protein lot numbers, use a premixed media. It also means that any pretesting is testing the whole culture environment, not just a part of it.

69.3.3 Toxic Oil

This is the big elephant in the room. Mineral or paraffin oil has allowed IVF to be performed in culture dishes rather than test tubes and has been the mainstay of all developments in the culture story. However, no one should be under the impression that oil is non-toxic and is actively associated with pH, gas and osmotic control and modifying the culture environment. As with media and protein, from a QC perspective, using oil supplied by the same supplier of media is the best option for several reasons. The primary one is that the media manufacturer has tested their media and additives in the presence of their own oil and the QC results for each batch are specific for that combination only. There is no guarantee that mixing oil and media from different suppliers will provide the same QC and will also certainly render void any responsibility of the supplier. One point to be made here is that supplier QC testing may be accepted as proof of quality, but a wise scientist would still confirm suitability in their own lab and culture system. There are stories that occasional sub-batches of some products may still fail, and failure to check the quality of all components on arrival is inviting difficulty if one needs to troubleshoot in response to a period of poor performance.

69.3.4 Culture Dishes

All culture ware is polystyrene plasticware, and many now are specifically tested for suitability for human IVF. The old systems using generic Falcon plasticware are long gone, but the newer dishes also must be treated with some caution. Issues such as duration of outgassing or rinsing prior to use should be clearly defined in the clinic policy manual and enforced with a signed checklist button by the scientist who sets up the next day's culture dishes. From a QC perspective that allows easy troubleshooting, a wise clinic would limit the number of types of plasticware used to the minimum number of items and then purchase a supply for culture dishes for a prolonged use, testing them against the current batch number prior to use. There have been anecdotal stories of items failing testing once, only to pass on a repeat test. This leads to another aspect that just because one packet of culture media is OK does not mean every packet of culture media is equally non-toxic. Supplier QC can only check a small percentage of items, and if there are within batch variation, then this may not be detected before shipping. This is why outgassing and/or rinsing has some appeal. It also raises questions whether all a client's embryos should be exposed to dishes from the same packet. One can easily become paranoid with such issues and in trying to spread the risk creating more confusion than required. The point is that if one couple embryos fail to develop or degenerate, then there is always the possibility of suboptimal quality in one dish among thousands.

69.3.5 Gas Mixture

Individual gas cylinders or specific gas mixtures are prepared in an industrial environment and dispute all precautions including stipulating only medical-grade gases, and where possible from a limited pool of cylinders, gas supply is one area that is not easily controlled. All one can do is to record when each cylinder is replaced and which delivery batch the cylinder came from. For additional cost, many suppliers can also provide a certificate of gas mix. The best way to manage gas complications is to use one or more in-line gas filters. These filters are largely activated charcoal and do have a limited life so as part of the QC manual is a requirement to follow the manufacturers' recommendations and change regularly.

69.3.6 Incubator Temperature or CO_2 Concentrations

The introduction of benchtop incubators has altered the complications arising from the use of large volume incubators with their inherent slow recovery times from each door opening and variation in temperature with location inside the incubator. Using large volume incubators for embryo culture requires considerably more QC management with the use difficult to document. Benchtop incubators while addressing such issues of recovery times, etc., suffer from problems in the actual monitoring of temperatures that the embryos are exposed to. They are also vulnerable to draughts and heat sources but do have the advantage that the location of each embryo cohort can be documented.

69.3.7 Warming Stages and Other Handling Environments

IVF has increasingly used heating stages on microscopes to try and minimise heat loss from the culture drops during handling and embryo manipulations. While a thermocouple can be attached to the stage near where embryo resides, it can be difficult to be clear what temperature the embryos are experiencing when there is a hole for light transmission in the middle of the stage. Even those with a glass plate still have temperature gradient issues. Ambient temperatures above the culture dishes give rise to the risks of draughts, etc. From a QC issue, all one can do is to document the temperature at least daily and make real-time monitoring available to all staff using the stages. Fully enclosed work stations solve many of these problems, but their use has failed to become mainstream in many laboratories. The author is an active supporter of their use.

69.3.8 Needles, Catheters and Other Handling Tools

While these tools are a necessary part of IVF, it is important to appreciate that they are sterilised with either ethylene oxide or equivalent and their lot number needs to be recorded for each case. In many laboratories, there may be many lot numbers of catheters and needles mixed together, making tracking of lot numbers unnecessarily complicated.

69.3.9 Environmental Issues (Perfumes, Deodorants, Renovations, etc.)

The risks associated with environmental pollution have adverse outcomes on IVF via the high exposure to volatile organic compounds which are well documented; other sources of variability may come from renovations and new equipment and motors, and personal items such as deodorants are far more difficult to note and manage. The use of air filters and positive air flows can alleviate some risks (but can also be the source of contamination). The use of air quality test monitors is probably a good inclusion in the laboratory particularly if the reading is included in a documented and auditable manner. An air quality KPI would be a very useful QC tool.

69.3.10 Staff and Personnel

Lastly, staff must be considered a key source of variability that is difficult to document and audit. Most clinics will review the pregnancy rate per staff member – both medical and scientific – but trying to track the contribution of each staff member to the success of a treatment cycle is nigh on impossible. There are several actions that the quality manager/scientific director could adopt. One is to enrol staff in a number of online quality control programmes dealing with identification skills (such as ► QAPonline.net). This will provide a tool to allow each staff member's visual assessment skills to be monitored. Second is to ensure each staff member is aware of and is abiding to the policies and procedure as set out by the clinic.

69.4 Knowledge and Awareness Is Power

If you have developed a set of rules for running the IVF laboratory as suggested above, then any circumstance that may result in a decrease of performance will hopefully pass quickly and with little noticeable effect. The central dogma is that there are a number of potential sources of "things" that may diminish the viability of gametes and/or embryos, but if you adopt an attitude of rapid recycling, then any such event will not significantly impact on your pregnancy rate. If your attitude is to do no harm to your clinic clients, then the best approach is to recycle quickly and minimise the exposure of detrimental components to as few clients as possible. You will never be able to identify such components, and their brief presence may only appear as a low point in the variability associated with your activity, but for me that is better than trying to prove something is toxic.

However, constant monitoring of the system will enable you to develop a set of information that you can use to both inform and alert you to potential downturns. Knowledge and awareness is indeed power, and all scientists should pursue this with vigour. Most clinical and nursing staff are focused on individual client's performance, and any information that you can supply on a regular basis will provide them with current information that can give to their clients and allow them to reassure them of the systems you have in place for their client's protection.

Knowledge is also important to alert clinical staff to trends that may affect the clinic's performance such as age ratios or number of embryos transferred and deflect their accusations that the laboratory is to blame for all clinical downturns.

To effect this, the clinic's database is your best friend as long as it allows you to interrogate any relationship. Excel spreadsheets and limited use databases may provide some limited KPI interrogation, but the clinic's plan should be to move to an information system with query capabilities.

69.5 Creating Tools to Manage the Clinic

This may be the critical section of this chapter. I have called the tools to monitor performance and troubleshoot possible complications KPIs, or key performance indicators ([3]). In reality all KPIs are tools to measure one or more parameters of an operation. The problem with IVF clinics is that many of the variables are unable to be controlled – for instance, the distribution of younger and older clients. So the KPI does not monitor production but provide guides to possible reasons why performance has changed. *After discussing KPIs, it is important then to identify the benchmark values for each KPI. KPIs only make sense when compared to their historical averages.*

In general, KPI may be divided into four categories:

1. Input clinical indicators (number and type of clients and treatment cycles started).
   (a) Number of new referral per time period (month) by age, previous history, needs (infertile, same gender, ovum donation, etc.), clinician referrals, etc.
   (b) Number of new treatment cycles per time period (monthly, quarterly, annually) by age (male and female), IVF × ICSI × PGD, previous history (previous attempts, attempt number, etc.), stimulated cycles × FET × ovum donation × sperm donation, PCOS × normal FSH × poor responder/ovarian failure (low FSH or AMH), etc.
   (c) Type of stimulation and FSH dosage regimens per time period

2. Gamete and embryo indicators
   (a) Number of oocytes/recovery/patient/time period (partitioned by age, FSH dosage, history, etc.).
   (b) Number of oocytes injected via ICSI/recovered/patient/time period/clinician. *This is a major KPI because the % injected/collected is a measure of oocyte maturation rate. It is also the "true" oocyte number/recovery. Many doctors will aspirate every follicle visualised, while others will only aspirate follicles with a reasonable chance of oocyte recovery.*
   (c) Fertilisation rate (FR) – by IVF × ICSI, 2PN × abnormal PN formation × sperm origins. *Using the ICSI true oocyte number for the time period, one can calculate the true IVF fertilisation rate. Note however that the IVF rate is often higher than the ICSI rate due to immature oocytes maturing after insemination and delayed fertilisation, giving a false impression of IVF superiority. One also measures alternative KPIs such as % failed fertilisation × all cases × cases with an acceptable oocyte number. Comparing FR from males with normal semen profiles to abnormal profiles to donor sperm profiles may also be of value. Sub-KPI may include:*
      (i) *# cases with no fertilisation*
      (ii) *# cases with no fertilisation with six or more oocytes*
      (iii) *# cases with abnormal fertilisation*
      (iv) *% abnormal fertilisations/fertilised oocytes*
      (v) *% FR for women≤35 and/or 38 years of age and six oocytes*
      (vi) *% FR for donor oocytes ≤35 and/or 38 years of age*
      (vii) *% FR for donor sperm cases*
   (d) Embryo cleavage – By IVF X ICSI × combined by day 1 2PN embryos. Variety of options including # 4 cell (day 2), A grade 4 cells (day 2), # 8 cells (day 3), # 7–9 cells (day 3), # A grade 8 cells (day 3), # viable blastocysts (day 5), # 4AA+ (best) grade (day 5), # viable blastocysts and 4 AA+ (days 5 and 6). *Note there are a number of combinations depending on how the clinic approaches day of transfer. These are all guides of embryo quality, and it does not really matter which one(s) one uses. The important issue is continuity. Any sustained decrease in the % viable embryos/2PN day 1 embryos may indicate either a decrease in oocyte quality or a change in culture conditions. Since embryo quality is always related to pregnancy rate, any decrease in % embryo quality should trigger an action response. It may be worth raising this at quality and/or management meetings since it may have associated costs to resolve.*
   (e) Usable embryos – by IVF × ICSI × combined × day of transfer (% of 2PN day 1 embryos and as % of oocyte numbers). This approach uses the definition of embryo quality as one that is suitable to transfer or cryostore. It suffers from lack of definition but in some ways actually reflects clinical practice. *Note this KPI has value in being one KPI to cover all laboratory activity and by and large can accommodate changes in clinical practice,* e.g. *does the clinic transfer cryostore on days 2, 3 or 5? Some clinic may transfer in day 2/3 but culture all residual embryos to day 5 or 6 to minimise the number of embryos going to cryostorage. Further, a clinic may have varying practices for younger or older women, egg number,* etc.
3. Pregnancy – outcome-based KPI.
   (a) % + ve hCG cases/transfer/fresh, frozen, combined. Each clinic should set their own hCG concentration for pregnancy depending on whether hCG is given as a luteal support, but 50 IU/l may be considered the lowest reasonable value to indicate if implantation has occurred. This KPI is an evolving one being updated with each pregnancy test. Some clinics do not test for pregnancy merely waiting to see if the client reports a pregnancy. However, most clinics are keen to ensure that all pregnancies are diagnosed and followed until clinical confirmation. *If your clinic has a relatively robust protocol for pregnancy diagnosis, then this KPI can be used as the first early warning tool for possible future issues. It can be used in-house to identify early pregnancy demise since clinical diagnosis may take another 3–5 weeks. Comparing fresh and frozen pregnancy and miscarriage rates (even if they are different modes) or the ratios of the two can be used as a KPI tool to identify major systemic issues in stimulation and/or culture or to issues with cryopreservation and recovery. Additional KPI may include:*
      (i) *Proportion of hCG values on the first day of testing above and below an agreed concentration, say <20 to >100 (or <40 to >100, you can decide any value). An increased incidence of low hCG indicates delayed implantation (either luteal support or poor embryo quality).*
   (b) % clinical (or ongoing) pregnancies/transfer/fresh, frozen, combined, donor oocytes, donor sperm. This KPI is a more stable statistic that is difficult to respond to and troubleshoot for since any issues that may have influenced a decease most likely will have past. For example, if a batch of media or oil was suboptimal, then by the time the total clinical pregnancies have been complied, eight or more weekly changes may have occurred. Furthermore, since only about 25–40% of cases will result in a clinical pregnancy, then the pool of cases will mean diagnosis is even more complicated.
   (c) % productivity rate [PR]. This KPI seeks to combine all the activity of clinics (fresh transfers, frozen transfers, freeze-all cycles, donors, etc.) and express the number of clinical pregnancies/treatment cycle (stimulated). Many do not understand this KPI since few use it, but for me, it represents what the clinic is all about – making babies against the main

activity (egg recovery). How many babies do you get per cycle? Regardless of how you manage your clinic – transfers on days 2, 3 or 5, PGD/PGS, and pro-cryostorage of blastocyst culture – it does not matter. What matters to the client is when all the embryos from any cohort have been transferred and what are their chances of having a baby. *The % PR is largely independent of how the clinic may change its activities. If the % PR falls, then you have a problem. If it rises, then any changes may have been positive. It does not work very well for age divisions.*

4. One further point about using KPIs for troubleshooting is more is not necessarily better. Select a suitable number to track regularly (weekly), others monthly, quarterly or annually. Don't confuse and overwork yourself with an excessive KPI list. Choose a limited number to present to monthly department or clinical meetings to both inform the clinicians and other staff and to demonstrate you are managing the system.
5. Having a number of KPI is fine, but to troubleshoot, you need to develop a set of reference ranges to identify potential issues.

69.6 Proactive and Reactive Approaches

Using benchmark reference ranges to troubleshoot.

The best way to use KPI is to develop a set or reference ranges with sufficient power to identify a mean and standard variation that is robust enough to identify if any single value is within statistical variability or exceeding the normal variation and therefore provides a warning sign of trouble.

The benchmark ranges need to be stable over time but capable of review being reset when appropriate. The most simple reference range is the previous year's activity. This should provide sufficient data points to give an appropriate estimate of variability and therefore be able to identify if any data point in the current years activity may be expected by chance alone or unlikely to be due to just random movement. The way to do this is to express it and every other data point as standard deviation (SD) units from the mean. This is known as the Z-score. If the mean value is say 10 units with a SD of 2, then a data point of 8 will be (8–10)/2 or − 2/2 or − 1. This means that the value of 8 is 1SD unit below the mean.

For example, if a basic KPI is % fertilisation rate (over all cases), then for every treatment cycle, there will be one data point. If the reference range for the previous year was 65% ± 25%, then an individual case of 75% will be (75–65)/25 or 10/25 or 0.4. In other words a 5Fr of 75% is entirely predictable, and you can rest easy. On the other hand, a %FR of 10% would generate a Z-score of (10–65)/25 or − 55/25 or − 3. It sounds easy but what does it tell you?

In effect once you have developed a reference score for any parameter, then you are able to place an estimate (in standard deviation units) of the likelihood of the value representing a normal fluctuation or a deviation from the routine fluctuations. It may allow you to write in a report if an outcome may signify an unusual event. But more importantly if you apply the Z-score function to two or more parameters, then you are starting to develop a set of tools to make troubleshooting decisions based upon statistical likelihood rather than knee jerk responses to a poor outcome. You can do this because you have rendered all the different values in a case (% mature oocytes, fertilisation rate, cleavage rate, good embryo rate, etc.) down to a common parameter.

Furthermore, you can aggregate data from individual cases to weekly means, monthly means, quarterly means and annual means. If the number of cases in any one aggregate is low, then you can operate the values as a combination mean of say the current and two previous periods. This has the effect of dampening the variability but allows for a better mean.

Figure 69.1 summarises all the information about how to use a Z-score profile. The plot is called a Levey-Jennings plot and can be applied to any circumstance where you have developed a set of data from which to draw a mean and standard deviation and data points is always presented in standard deviation units from the mean. Its primary function is to examine consecutive data points (% fertilisation cases) or monthly means or any other format and seeks to predict if the data set reflects a system under normal operating control or whether the system in not operating under normal control. There are a set of rules called the Westgard rules that can be applied to detect operating failure or ransom variability [5, 6]. In other words, if one uses say % fertilisation as the key KPI, then Westgard rules may be a tool that you can use to anticipate any deviation or set of deviations within normal operating variability or a reflection of dysfunction requiring you to inform others and to act to correct the problem.

Figure 69.1 illustrates several key aspects to this tool. These are:

1. Each point on the graph is an individual client's result (in this case it is % fertilisation rate) – each expressed as a Z-score (standard deviation from the preset mean).
2. They are all centred around the mean of the preset values.
3. Some are more than the mean, and some are less than the mean, i.e. tools can determine if a value is an increase or a decrease.
4. In these cases, about half the values are above the mean and half are below the mean. This is good in that it tells you that the mean is about correct for the current data set and the system (your laboratory) is functioning normally.
5. The two blue areas (two light blue and two dark blue) can be defined a Z-score range where you may wish to consider action. This tool can then provide you with a set of values that may indicate abnormal activity – the light blue may act as a warning – "keep an eye on the system", while the blue area may indicate you need to act in some prescribed manner. These warning and action cut-offs can be set by you to any value you consider appropriate, but in these cases, they are 2 SD and 3 SD deviations from the mean.

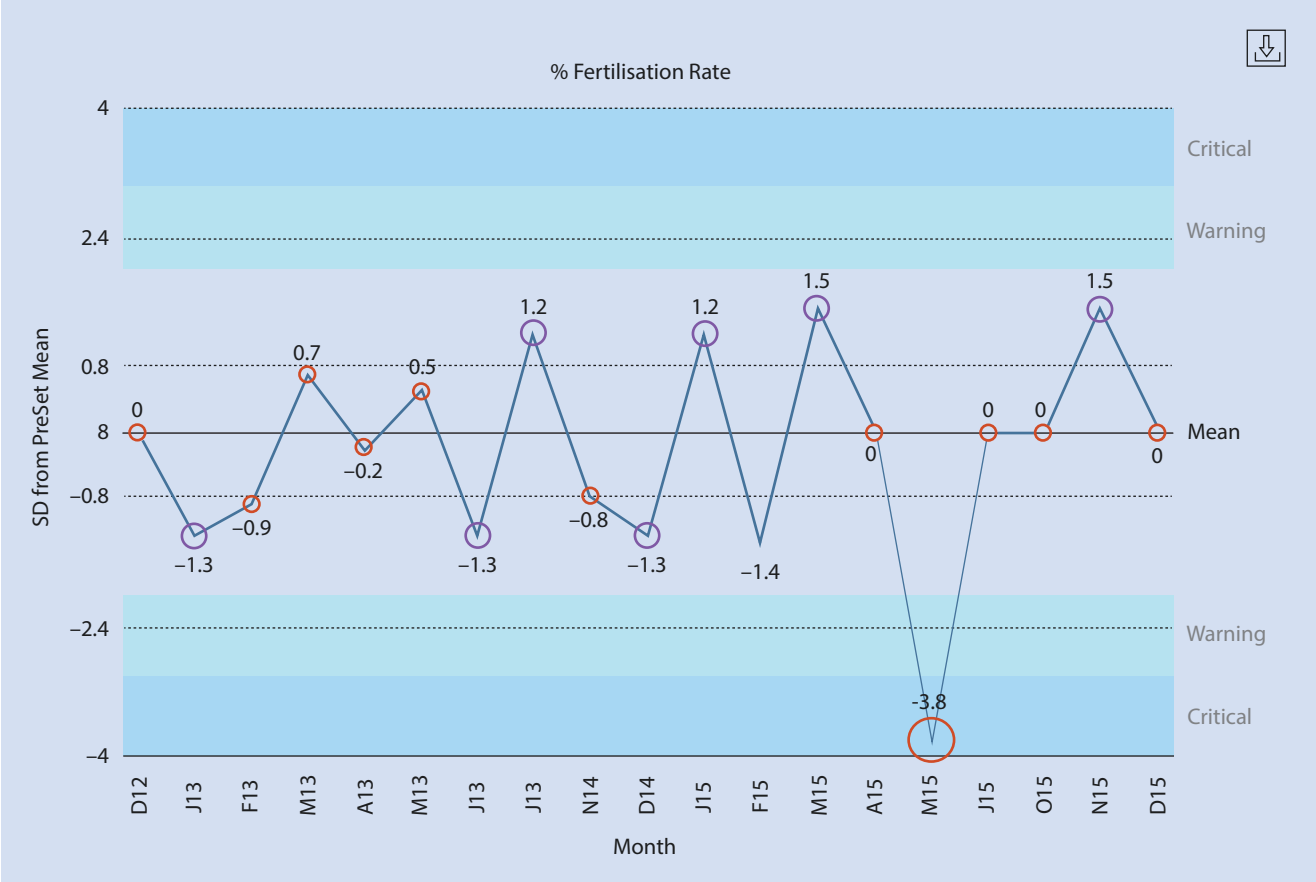

Fig. 69.1 An example of how to use the Z-score

6. Each value is represented by a dot of varying sizes. These dots indicate if the value is within 1 SD, 2SD or 3SD unit from the mean.
7. One value (M15) is more than 3SD units below the mean. The probability of his happening is less than 1/100 and may be interpreted as an abnormal event.
8. However from a troubleshooting perspective, it is a once-off event – more likely related to patient parameters than to a systemic laboratory event. *This underlies a critical difference to how Z-scores are used in an IVF setting compared to an industrial setting. In an IVF laboratory, there is always the chance an abnormal event (failed fertilisation) may be due to client medical factors (abnormal morphology, poor oocytes) than due to, for example, poor culture medium.*

69.7 Tools to Understand Sources of Variation and Risk

This last point is the hub of troubleshooting in an IVF laboratory. Does any individual outcome signify a change in laboratory quality or an individual client's dysfunction? How many bad outcomes do you need to record before you raise concerns and before you act? Remember that IVF is about treating clients with reproductive deficiencies and any failure may not be due to laboratory problems. Remember also that any issue (say poor media or oil) may well pass through the system before you can determine if the problem is persistent. They do require the data set to be normally distributed which some IVF KPI are not. In essence, 66% of all cases will lie within 1SD of the mean, 95% within ~2SD of the mean and less than 1% outside of 3SD from the mean. The rules basically look at how consecutive cases distribute themselves around the mean and look for combinations that are unlikely to occur by random chance. The rules were designed around biochemistry instrumentation and control testing, and their application to IVF is not the same where each data point (e.g. % fertilisation of patient X) is a mixture of many elements. The nett effect of this is that the standard deviations are usually quite wide, and therefore any deviation needs a significant one. The point of applying the Westgard rules is not to look at any one value but the trends in the distribution rolling forward with each case.

Notwithstanding these caveats, the Westgard rules (see ► www.westgard.com/mltirule.htm) are a useful stating point. In summary these include:

(a) 1_{3s} _ any case where one value is more than 3 SD from the mean [warning]

(b) 1_{2s} _ any case where two values are more than 3 SD from the mean [warning]

(c) $2_{2s\_}$ any situation where three values are more than 3 SD from the mean [action]
(d) R_{4s} – any situation where one value is <3SD from and the next is 3SD > mean [action]
(e) 4_{1s} – any situation where four values are more than 1SD from the mean on the same side of the control mean line [action]
(f) 8×, 10×,$12_{\times}$ – any situation where 8, 10 and 12 consecutive samples fall on one side of the mean regardless of SD from mean [action]
(g) $2of3_{2s}$ – any situation where two out of three KPIs exceed +2Sd or −2SD for any one month or any period) – multirule [action]
(h) 3_{1s} – any situation where three KPIs exceed +1Sd or -1SD on the same side of the control line for any one month or any period – multirule [action], e.g. % mature oocytes, %fertilisation rate, % utilisation rate
(i) $6_{\times,}9_{\times-}$ – any situation where between six and nine KPIs are on the same side of the control line for any one month or any period – if you have that many.
(j) 7_{T} – any period where a KPI SD from the mean incrementally changes over time in the same direction, e.g. from -2Sd to +2SD, each getting bigger.

Clearly one may go crazy trying to implement all these guidelines, but from a troubleshooting perspective, they are pointers to whether you need to look more closely at what is happening in the laboratory. A warning trigger may merely cause you to look at the last cases moving through the laboratory, while an action trigger may require you to look more closely at the cases, looking for changes in media, gases or equipment, and at staff (clinical and scientific). It will help enormously if the pre-emptive measures discussed above are implemented. For instance, I have always liked starting new media bottles each week (you can do it fortnightly if you are a big clinic) because you can look at the outcome of each case from Monday to Sunday. Have the log of when new plasticware or gases are introduced. Sometimes it is best just to talk to the staff or comments. There is where a permanent activity in the laboratory by all quality managers is a good thing since it keeps you in touch with the embryo quality.

Remember, IVF is both a robust technology and a fragile technology. There may well be many aspects that are suboptimal, but with a system that cycles lot numbers quickly through the laboratory, any adverse effect will be minimised. Most IVF equipment is pretested using the mouse embryo bioassay (MEB) which is good, but this is a very low-level quality tool. A set of plasticware may well pass the MEB but still possess some low-level toxicity. My strongest suggestion is to look at embryo quality, both rates and uniformity of cleavage and degree of fragmentation. While one may expect many embryos to be of less than perfect quality, there should be at least one or two embryos of A grade quality. If your staff are subject to routine quality assurance testing (as at ► www.QAPonline.net), then this is the best guide to laboratory quality.

Attempting to apply these rules in-house can be challenging, and some databases may provide for such analysis. There is one online, anonymous tool (► www.IVFbenchmark.com) that is available where the trends (monthly) are reviewed against the Westgard rules dynamically.

69.8 Communication Is the Key

If a set of KPIs that reflect the clinics activity are presented on a regular basis (monthly, quarterly), the QC manager is able to provide information to allow all senior staff to be aware of variations in activity and quality to ensure that any perception of a loss of performance is addressed. This has the effect of limiting unnecessary and time-consuming disruptions. Performance reports by medical and scientific staff are high on this list as most see this information personally. Using a Levey-Jennings approach to manage these KPIs will provide you with a case-by-case spyglass into the operation of the clinic and the laboratory and provide you with a response from clinic managers about quality and provide you with a rapid warning of change that may or may not warrant major action. Good luck.

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Benchmarking and Decision Making in the IVF Laboratory

Julius Hreinsson

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Learning Objectives

- Learn the most common KPIs for the IVF laboratory and their values
- How to monitor the KPIs over time and how to interpret variations in them
- How to apply the above for troubleshooting in the IVF laboratory
- How to improve decision making in the IVF laboratory

70.1 Introduction

The concept of benchmarking or key performance indicators (KPIs) is well known in many operations in science, medicine, and business. It is not possible for a manager in any business operation to run her unit without monitoring, for example, EBITDA (earnings before interest, taxes, depreciation, and amortization). This principle is an integral part of any business management, and the laboratory KPIs must be considered in the same way. KPIs are also an important component of quality control systems such as the ISO accreditation standard ISO 15189 (▶ www.iso.org). The basic concept behind using KPIs is to collect information on the operation, and the information is then used as a basis for decision making. It cannot be sufficiently stressed that in order to achieve good-quality decision making, objective facts must be used as a basis for taking decisions. In this chapter the application of KPIs will be discussed, including how to select and apply them and also how to use them to achieve high-quality decision making in the IVF laboratory and thus to improve results.

This chapter aims at giving an introduction to the concept of KPIs and their use in the embryology laboratory as well as some aspects of decision-making processes. Focus is on the most important KPIs and their relevance for day-to-day activities. This chapter does not aim at listing all conceivable KPIs, and for a more thorough and detailed coverage, the reference list gives suggestions for further reading.

70.2 Key Performance Indicators (KPIs)

It is not possible to run a successful IVF unit without regularly checking the results of the work being performed. The first and most obvious parameter to monitor is the pregnancy rate for the clinic. This is of course important to know; it is usually the first thing a patient would wish to know, and it is the prime parameter of interest for clinicians – of course! The intended outcome of the activities is a healthy baby for the patient, and the rate of achieved pregnancies in the clinic per oocyte collection or embryo transfer is a surrogate marker for this goal.

The laboratory director, however, will soon realize that the clinics' pregnancy rate is not very useful for everyday decision making. Although interesting and important, the pregnancy rate has several disadvantages as an outcome parameter, including the following:

- The delay from the time of activity until results show up is approximately 3 weeks – at least.
- Results from several weeks or months must be collected to reach any statistical significance.
- Poor culture conditions may be partially masked by replacing several embryos at a time.
- Pregnancy rates from fresh transfers say nothing at all about embryos/oocytes which may have been eligible for cryopreservation.
- Pregnancy rates say little about other important aspects such as fertilization rates, canceled cycles, and other aspects relating to pre-transfer efficacy.
- The embryo transfer itself may – if improperly performed – ruin the results for a perfectly good embryo laboratory.
- Pregnancy rates do not allow any analysis of which processes in the laboratory may be suboptimal and how far they are from acceptable ranges.
- Accumulation of patients with poor prognosis during a period of time will lead to a drop in results without any faults in the laboratory.

Too often, decisions are made in IVF laboratories based on short-term changes in pregnancy rates without a proper analysis of the underlying reasons. This will most likely lead to poor-quality decision making and is not likely to lead to any long-term improvement.

If, on the other hand, the laboratory can ensure that all processes are functioning normally, then poor results need to be studied from the clinical perspective.

70.3 What Can Be Gained from Using KPIs

The main reason for measuring KPIs in the IVF laboratory is to ensure high performance and reproducibility in the daily processes. By correctly defining these parameters and ensuring that laboratory outcomes remain within acceptable levels, the probability of good results increases dramatically. Monitoring the laboratory activities by using KPIs will also increase the level of control of the laboratory activities, and proper use will aid in finding areas which need improvement. In fact, without KPIs, we are not accurately able to determine whether the program is on course.

70.4 Important KPIs for the Embryologist

Relevant and recommended levels of KPIs in assisted reproduction have previously been presented [1–3]. At the end of the day, the individual laboratory needs to define which parameters are relevant for its daily operations and which levels can be attained. Issues such as the patient population treated, embryo transfer policy, etc. need to be taken into consideration. At the same time, certain KPIs and their levels may be considered universal. Also, it is common to define

Table 70.1 The table specifies the most important KPIs for the embryologist/laboratory director to monitor

	KPI value (%)	Benchmark (%)	Frequency of monitoring
Oocyte recovery rate	80	90	Biannually
Oocyte maturity	75	85	Annually
Degenerated oocytes at recovery	3	1	Biannually
Sperm recovery rate	20	40	Biannually
Fertilization rate	60	70	Monthly
Polyspermy rate	5	3	Monthly
Poor fertilization rate IVF	10	5	Biannually
Failed fertilization after IVF	3	2	Biannually
ICSI damage rate	10	5	Monthly
Poor fertilization rate after ICSI	5	2	Monthly
Failed fertilization after ICSI	3	1	Monthly
Frequency of 1pn after ICSI	5	2	Biannually
Embryo cleavage rate	95	99	Monthly
Early cleavage rate	10	15	Biannually
Proportion of abnormal cleavage in time-lapse	10	5	Biannually
Blastocyst development rate days 5–6	40	60	Monthly
Good-quality embryo rate days 2–3	30	45	Biannually
Good-quality blastocyst rate days 5–6	25	40	Biannually
Embryo utilization rate	40	60	Biannually
Survival after cryopreservation/thawing	80	95	Biannually

The KPI value is the minimum acceptable level (or maximum acceptable level for "deviation" parameters such as failed fertilization). The benchmark value is the aspirational level to attain. Further details are given in the text

two levels, first the minimum acceptable level and second the aspirational level the laboratory strives to attain.

The minimum acceptable level is the point below which the process must be considered to have a problem which needs immediate attention. If the problem is not identified immediately and preventative measures taken, the process must be discontinued until the problem has been solved. The aspirational level on the other hand is often considered to be the "benchmark" of the process, i.e., the level at which the process is considered to be running optimally according to current best practice and knowledge.

The frequency of monitoring of the KPIs is important to define since it may not be meaningful to monitor all of the parameters at the same frequencies. Table 70.1 gives details on some 20 important KPIs for the embryologist, and although it should not be considered to be a definitive list, it contains most of the important parameters which the embryologist needs to pay attention to. Further details are explained below.

70.4.1 Oocyte Recovery Rate

Rate of identification of a cumulus-oocyte complex per aspirated follicle at oocyte collection.

70.4.2 Oocyte Maturity

Rate of mature (MII) oocytes per total number of oocytes collected.

70.4.3 Degenerated Oocytes at Recovery

Rate of damaged or degenerated oocytes per total number of oocytes collected.

70.4.4 Sperm Recovery Rate

Yield of motile sperm after preparation, i.e., the number of motile sperm cells obtained after preparation per total number of motile sperm in the sample.

70.4.5 Fertilization Rate

Rate of fertilized oocytes with two pronuclei (2 pn) per total number of inseminated oocytes.

70.4.6 Polyspermy Rate

Rate of oocytes with three or more pronuclei per total number of inseminated oocytes.

70.4.7 Poor Fertilization Rate After IVF

Proportion of treatment cycles in which <25% of inseminated oocytes were fertilized with 2 pn.

70.4.8 Failed Fertilization After IVF

Proportion of treatment cycles in which none of the inseminated oocytes were fertilized with 2 pn.

70.4.9 ICSI Damage Rate

Proportion of injected oocytes by ICSI which degenerate at any stage of manipulation, from denudation to microinjection until fertilization check.

70.4.10 Poor Fertilization Rate After ICSI

Same definition as for IVF above.

70.4.11 Failed Fertilization After ICSI

Same definition as for IVF above.

70.4.12 Frequency of 1pn After ICSI

Proportion of microinjected oocytes displaying 1 pn at fertilization check.

70.4.13 Embryo Cleavage Rate

Proportion of zygotes (2 pn only) which cleave to become embryos.

70.4.14 Early Cleavage Rate

Proportion of zygotes which have cleaved at the time of early cleavage check.

70.4.15 Proportion of Abnormal Cleavage in Time-Lapse

Proportion of zygotes displaying 1–3 cleavages, 2–5 cleavages, or reverse cleavage, i.e., frequency of abnormal cleavage.

70.4.16 Blastocyst Development Rate Days 5–6

Proportion of zygotes which are at the blastocyst stage 5–6 days after insemination.

70.4.17 Good-Quality Embryo Rate Days 2–3

Proportion of embryos with good-quality morphology at evaluation according to the laboratory evaluation system.

70.4.18 Good-Quality Blastocyst Rate Days 5–6

Proportion of blastocysts with good-quality morphology at evaluation according to the laboratory evaluation system.

70.4.19 Embryo Utilization Rate

Proportion of embryos (cleaved zygotes) transferred or cryopreserved for the patient.

70.4.20 Survival After Cryopreservation/Thawing

Proportion of embryos utilizable after cryopreservation/thawing.

70.5 Interpretation of KPIs and Correlation with the Clinical Activities

By using the KPIs to measure the efficacy of the various processes in the IVF laboratory, it becomes possible to identify potential improvement and to aid in troubleshooting at various stages. There may be several interpretations for the problems which may arise in the IVF laboratory. Below we give two examples of how KPIs may be used to assist in diagnosing problems and troubleshooting.

70.5.1 Poor Results in ICSI Cycles

The potential reasons for poor or dropping ICSI results can be many. For example, this may be caused by improper selection of buffer media for injection, faulty calibration of the warming plate, etc. The possibilities are numerous. This may also be a purely technical/manipulative issue with the cause residing in individual operators. One way of approaching such a problem is to look at the individual KPIs and look for clues.

First, a look at the rate of degenerated oocytes during denudation or microinjection will show whether these processes are a problem.

The rate of 1pn oocytes after ICSI will show whether sperm immobilization is an issue.

The cleavage rate of oocytes after ICSI and good-quality embryo rates will show whether the culture media system is performing properly.

By studying the KPIs, we can isolate a likely cause of the problem. For example, if degeneration rates are low (<5%) and cleavage rates are high but good-quality blastocyst rates are low, then perhaps the long-term culture incubator needs to be recalibrated. And vice versa if oocytes degenerate at a high rate (>10%) but those oocytes which do fertilize cleave normally, then probably the denudation or injection processes need to be analyzed further. If all laboratory KPIs are normal or high, then possibly the issue is outside the laboratory.

The important issue here is to have reliable data on the performance of the laboratory on which to base decisions regarding further actions.

70.5.2 Treatment Cycles with No Fertilization

High rates of cycles (>5%) without 2pn fertilized oocytes may be caused by various suboptimal conditions or processes in the laboratory. The most obvious cause could be that the selection of patients for ICSI could be improved but other more subtle reasons can have an effect. For example, if the aspiration pressure at oocyte collection is high (>120 mm Hg), this may damage oocytes, especially oocytes from older patients, and increase risk for failed fertilization cycles. The rate of damaged oocytes at collection should then be very low (see Table 70.1).

Another possible cause in this instance is that the insemination concentration may be low. In this case, an overall low fertilization rate should also be observed.

70.6 Control Charts

Another way to use the KPIs is to monitor and document these over time and plot them against time in a chart, creating a so-called control chart (Fig. 70.1). This allows us to identify slow changes in the indicators and will give additional information regarding laboratory performance. If conditions in the laboratory are slowly changing for the worse or for the better, these charts will give important information on these tendencies and will allow preventative action to be taken in case of deteriorating results.

Sometimes it is not clear whether the laboratory or the clinic is performing suboptimally. In these cases, if laboratory benchmarks are at high levels and control charts show stable performance, then clinical factors should be studied if pregnancy rates are low. The opposite may also be the case, of course. Interlaboratory controls, as specified by the international accreditation standard ISO 15189, are of course highly valuable to calibrate the IVF laboratory against international operations in the same field.

Control charts will also allow diagnosis of potential issues with instruments, such as temperature variations in incubators. Incubators have a recovery heat impulse after the door to the incubator has been closed. If the impulse is not correctly calibrated in terms of time or effect, unnecessary temperature variation will be observed. Figure 70.2 shows an example of such a situation.

70.7 How to Deal with Patient Variation Regarding KPIs

It is well known that differences in the patient population between clinics are an important cause of variation in IVF results [4]. The most important prognostic factor for IVF success is still female age at the time of treatment, but other factors such as number of previous treatments, source of sperm, and numerous others will also have an impact. It is therefore important to consider specifically monitoring KPIs for certain groups of patients, for example, women <38 years of age. KPIs for specifically defined groups of patients should remain relatively stable, while the total population of patients presenting at the clinic may vary over time. The laboratory and embryological parameters are relatively stable over various patient groups, but it is not reasonable to expect the same results from patients over 40 years of age and those who are under 35 years, for example. Therefore, when making decisions regarding whether a certain KPI is above or below an acceptable level, it is important to double-check the results when, for example, only patients under a certain age are included in the data collection.

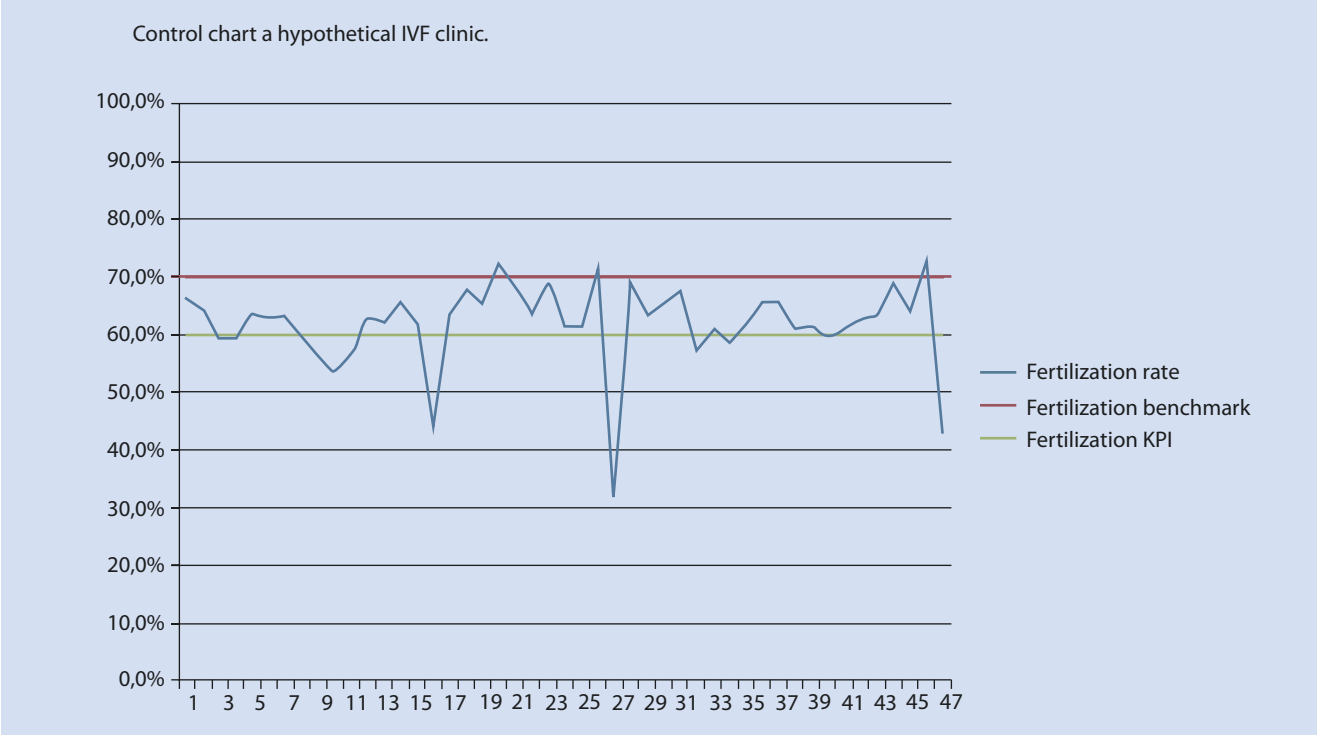

Fig. 70.1 Control chart from a hypothetical IVF clinic. Example of a control chart from a hypothetical but typical IVF clinic. The diagram shows the monitoring of fertilization rates per week during the year. The results are above the KPI most of the time, with sporadic dips in results but no obvious trend visible from the data. On the other hand, results in some weeks exceed the benchmark, often in close proximity to dips. The most logical explanation is that the laboratory has a sound culture system with acceptable fertilization rates but that, during some weeks, results are affected by patients with poor prognosis

70.8 Decision Making

In a situation with incomplete knowledge and a weak factual basis, decision making is difficult. A high workload, stress, and lack of time for reflection coupled with a hierarchical organization increase the risk of poor decision making. It is well known that in health care in general, poor decision making and a lack of communication are the underlying causes of mistakes, which all too often may lead to injury or have fatal consequences. International studies show that rates of medical injury range from 10% to 13% for hospitalized patients. Many of these problems are the result of cognitive mistakes but rarely because of technical or training mistakes or because of lack of knowledge [5]. Errors in medical diagnosis have been identified by several authors as an area where cognitive factors may be involved [6, 7]. From an organizational perspective, it is also clear that such a problem must be addressed in a systematic fashion and not just to be blamed on the individuals involved. Higher levels of security in health care are reliant on transparency of methods and secure systems allowing high-quality procedures. Involving the patients themselves in medical decisions is also considered helpful.

Table 70.2 gives an overview of the most common heuristics and biases along with a very short explanation of each one. Awareness of these issues has been established in safety culture industries such as aviation and nuclear power plants and recently in some areas of the medical literature. Focus has often been on the individual biases and errors, however, and a comprehensive theory on these issues is not prominent in all areas of the medical literature. Fortunately, however, some exceptions to this general rule can be found although they may not be very well known. Sadly, medicine is hardly to be considered a safety culture industry: if, for example, an airline company were to operate at a serious error rate of 9% per flight, then they would be out of business immediately!

Heuristics are useful in situations where decisions must be taken quickly and there is little time for reflection. It is crucial to realize that it is exactly in these situations when cognitive biases may have an effect, especially when many parameters must be evaluated simultaneously. Additionally, as medicine is a very complex system with a multitude of input parameters, it is easy to fall prey to biases such as feedback bias, where absence of feedback is taken for positive feedback. The reason may simply be lack of patient continuity or as in IVF where we never know if embryos which are deselected had the potential to lead to the establishment of a pregnancy. Another prominent opportunity for bias is confirmation bias where information on which decisions are based is selected according to the decision maker's opinions. A complete description of these biases and heuristics is beyond the scope of this chapter.

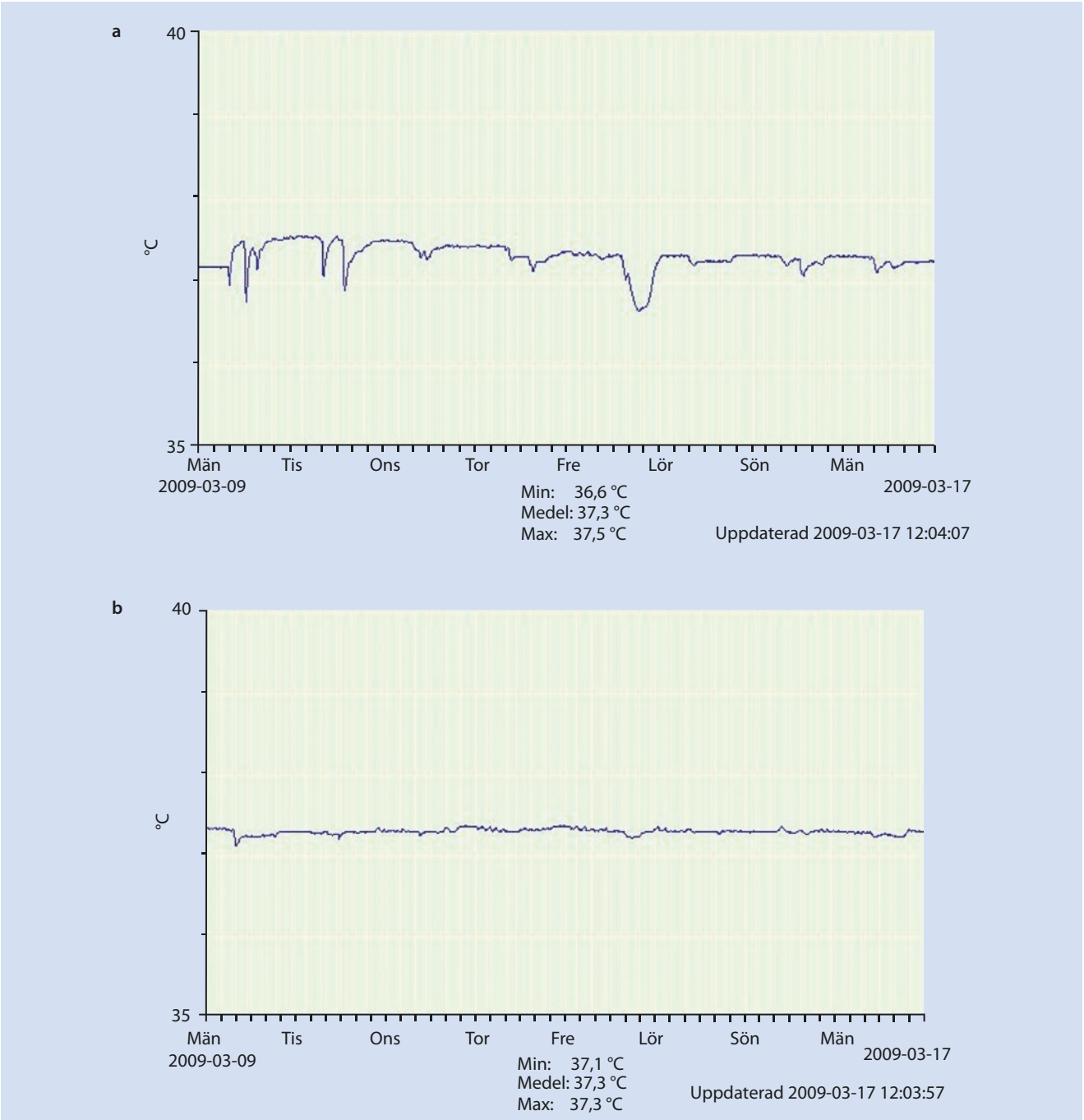

Fig. 70.2 Temperature monitoring in incubators. The charts show temperature monitoring in two incubators over a period of 8-day activity in an IVF laboratory. Incubator in 2.a has a poor regulation of the heat impulse after the incubator door has been closed

There are many ways to improve decision making. Knowledge of the abovementioned biases is the first step toward improvement. The use of information technology is a possible way to improve decisions in complex situations, for example, the use of algorithms and modern data processing techniques. Even the use of simple linear models may improve decision making in medicine. One interesting technique is to take an outsider's view on the situation or to externalize decisions in some way. However, the task of changing decision-making processes is a huge effort and one that is both very difficult and probably unappreciated since here we are working with thought processes which the individuals themselves usually are not aware of.

Poor decision making in the IVF laboratory is not likely to lead to direct harm to the patient but may decrease the probability of treatment success. Too often in the IVF laboratory, decisions are made on poor grounds, which almost inevitably leads to suboptimal results or failure to solve the problems at hand.

Table 70.2 An overview of heuristics, biases, and cognitive errors in decision making

Heuristics	Biases	Short explanation of each
Availability heuristic		Assessing frequency, probability, or cause by degree of availability from memory
	Ease of recall	Memories and recent experiences affect judgment
	Retrievability	Assuming available recollections are representative of a larger pool of samples
Representativeness heuristic		Stereotyping
	Insensitivity to base rates	Failure to plan for adverse outcomes
	Insensitivity to sample size	Draw conclusions from poorly powered studies
	Misconception of chance	Overestimate generalization of empirical findings
	Regression to the mean	Predict based on past data. Overinterpret statistical outliers
	Conjunction fallacy	Combination of descriptors is judged more probably than any one component
Positive hypothesis testing		Congruence heuristic. Assume a statement is true
	Confirmation trap	Evidence confirming own opinion are more highly evaluated than disconfirming evidence
	Anchoring	Initial evaluation is hard to adjust
	Conjunctive/disjunctive bias	Underestimating combined frequencies of independent events
	Overconfidence	Overestimating chance of success
	Omission bias	Tendency toward inaction for fear of causing harm
Affect heuristic		Emotional influence on decision making
Bounded awareness	Premature closure	Decision reached prematurely – Before all facts are examined
	Feedback bias	Misinterpretation of no feedback as positive feedback
Framing	Framing bias	Initial presentation affects subsequent thinking
	Status quo bias	Bias against action
Motivation and emotion	Positive illusions	Viewing the world in a more positive light than is objectively accurate
	Self-serving reasoning	Situation is perceived differently depending on the individuals' role
Escalation of commitment		Persisting on a previously determined course of action
	Sunk costs	Unwillingness to abandon a failing decision, coupled to ego
	Escalation paradigm	Analysis of forces affecting escalation of commitment

From: Refs. [5, 8]

70.8.1 Basis for Decisions

It is crucial to base decisions on facts. Everyone has opinions, and too often dominating individuals with strong views on the subject at hand may have a large influence on decision making. Facts can be collected in various ways. This chapter is on KPIs, and this is of course one way of collecting facts. Scientific literature and published data are extremely important in this respect. Informed strategic and tactical decisions for the laboratory must be made based on scientific and factual evidence.

One example of a situation in the IVF laboratory where published literature can be of assistance is the question whether it is beneficial to collapse blastocysts before vitrification. The individual laboratory may have limited time or resources to put into testing this issue completely, so the scientific literature needs to be used as a source of facts on which to base a decision on this issue. New and older publications show conclusively that improved cryosurvival is attained by collapsing blastocysts before vitrification [9, 10]. If the individual laboratory decides not to collapse blastocysts before vitrification, it is necessary to realize that this is then for other reasons than actual cryosurvival or hopes for better results.

70.8.2 Nontechnical Skills

Bias in decision making is an important reason for mistakes in health care as mentioned above. Many of these problems are the result of cognitive mistakes but rarely because of technical or training mistakes or because of lack of knowledge. Originally, attention was first turned to this type of problem in the aviation industry where several tragic accidents in the 1970s were traced to bias in decision making and which later led to the development of countermeasures in the form of training in nontechnical skills [11]. These skills are to a high degree utilizable in health care, especially in emergency situations, but also in situations of incomplete knowledge and complex and multiple data inputs. This is true for typical situations in the IVF laboratory; therefore a mention of nontechnical skills is relevant here.

According to Flin et al. [11], the most important nontechnical skills are the following:

- Situation awareness
- Decision making
- Communication
- Teamwork
- Leadership
- Managing stress
- Coping with fatigue

To some degree this applies to the situation in the IVF laboratory. It is, for example, important to realize which situations are most likely to lead to poor decision making, for example, during situations of stress or pressure in the laboratory. One of the most important issues here is to develop the ability to increase the quality of decisions by establishing routines for and facilitating shared decision making. This means simply to invite colleagues to discuss difficult issues from various points of view. Morphological evaluation and selection of embryos for transfer or cryopreservation are a typical issue where it can be very useful to improve the quality of decisions by shared decision making. Decisions on the issue of embryo selection may have several aspects separate from simply increasing the chance of pregnancy. There may be economical issues and medical situations to consider, one or two embryos to transfer, etc. One way to facilitate shared decision making in situations of limited decision-making time is to document routines for how to act in various circumstances and to use a systematic approach as far as is possible. This allows input from colleagues while facilitating quick and efficient work with the added value of opening up the possibility of process evaluation.

Developing a permissive atmosphere in the IVF laboratory is also important, and developing a situation where the IVF laboratory becomes an organization which facilitates learning and development must be the goal of management.

Table 70.3 Classification of an organization's ability to learn and improve its processes

Low ability	High ability
Difficulties or problems are hidden and considered signs of failure. Scapegoats are found	Problems are analyzed and discussed to keep the organization in a state of "continuous crisis"
Only attempts to change previously approved by the "center of power" are approved	Experimentation is encouraged
Excuses are found for failures	"Failures" are considered highly interesting and lead to new experiments
Rewards are given mainly with consideration to loyalty to management and tradition	Rewards are given to those who show new problems and solutions
Structure of power remains unchanged	Continuous displacement of power

70.9 The Learning Organization

The ability to change an organization is crucial to maintain long-term viability, and this is to a very great extent true of the IVF laboratory. New techniques are developed at a fast pace, competition is continuously increasing, and new demands from authorities and customers need to be considered. At the same time, opposition to change is a natural and normal reaction in any organization. The magnitude of opposition will depend on the nature of the proposed change, and sometimes opposition is part of shared decision making. Not all changes are beneficial, perhaps insight is limited, or timing may not be optimal for various reasons. It is necessary to be aware of three types of inertia as regards organizational changes: organizational inertia, insight inertia, and maneuvering inertia. It does not do much good if the organization realizes that changes need to be made but is incapable of implementing them [12].

The learning organization is distinguished by continuous development and improvements through learning. The learning is achieved through experience and feedback from others. It is important to create an environment characterized by tolerance and allow individuals to try new methods and approaches – to allow mistakes. This is not always easy in the strictly regulated environment of the IVF laboratory, but it is an important concept. Individual learning is then transformed to organizational learning through communication and quality systems.

A given organization may be considered to have low or high ability for learning, depending on how it reacts in different circumstances. See Table 71.3 (modified from Ref. [12]).

We need to remember that participation of those concerned is necessary to effectively change an organization because insight and information that a change is necessary are

important. Change is usually difficult in the beginning, but the processes become easier as time goes by – after the initial threshold is passed. The important issue is that the embryologist is a knowledge worker and it is crucial to approach work in the embryology laboratory in a professional way.

70.10 Conclusion

The IVF laboratory should be considered a learning organization, a center for the development of knowledge, not just a factory which provides routine services. In this way we will be able to allow the development and improvement of our daily work. One important step in this direction is to assure a professional attitude and processes in the laboratory. To assure this, proper decision-making processes must be applied. Decision making should be an inclusive process, especially in complex situations. Second opinions should be sought whenever necessary. Additionally, decisions must be based on facts – not hearsay or opinions. If we don't have the facts – we need to obtain them! Here, benchmarks give us a tool to evaluate our work and build on our own experience and that of others.

We should not accept lower standards than any other laboratory, and it is important not to put too much weight on pregnancy rates as an indicator of performance. Reality in the embryology laboratory is far too complex to rely on one quality control indicator as an adequate measure of the daily processes.

Review Questions

1. How would you interpret a low value for cleavage rates in the embryology laboratory? Give examples of possible explanations and how to use the KPIs to find the root of the problem.

2. The embryology laboratory has a high rate of degenerated oocytes after ICSI. What is a possible explanation for this and which steps can be taken to improve the situation?

3. What steps can be taken in everyday work to improve decision making in complex and stressful situations?

4. Is your place of work a learning organization? Why/why not?

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Innovations, Risks and Safety in ART

Contents

Automation, Artificial Intelligence and Innovations in the Future of IVF

Alex C. Varghese and Charalampos S. Siristatidis

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Learning Objectives

- To learn about the novel developments in automation and robotics related to IVF laboratories
- To understand the need of prediction models in the IVF setting
- To get familiarized with the development in IVF lab
- To understand the gaps related to the low rates in IVF
- To see how artificial intelligence works and how it can be implemented in the IVF setting

71.1 Introduction

The miracle technology in the medical field "in vitro fertilization (IVF)" which so far resulted in the birth of more than 5 million babies worldwide has gained momentum across the geographies as one of the best treatment options for infertility or childlessness [1]. A recent calculation on the demographic projection of the contribution of assisted reproductive technologies to world population growth indicates that an estimated 157 million people alive at the end of the century will owe their lives to assisted reproductive technologies (1.4% of global population) [2]. Even WHO has recently declared infertility as a disease. Compared to the scepticism by layman and even by medical professional in the early years of IVF, it has flourished well and now an established discipline in medical sciences. Almost all the countries worldwide have either fertility or advanced ART clinics to cater to its childless couples. IVF lab is unlike the other medical laboratories considered a workplace for human cell therapy, a very recent category of laboratories complying with the demands of regulatory authorities [3]. The assisted reproduction technologies (ART) labs provide resources for all steps of IVF treatment: gamete handling, IVF or micromanipulation, embryo culture, preparation for embryo transfer, and cryopreservation. Moreover, auxiliary laboratory resources are often integrated to the IVF lab, such as andrology, hormone analysis, quality control, and molecular genetics (PGD/PGS).

The early pioneers of in vitro fertilization who were trained in research or cell and tissue culture methods adopted the systems from similar field to cater to the needs of the embryo culture. Hence early days of IVF laboratories and methods were exact replicas of cell culture labs. As the research evolved on the optimization of culture conditions, newer embryo culture systems and procedures were introduced at a slower pace. "Slower pace" in development was typical across the IVF labs which may attribute to the scepticism or fear of introducing failures in outcomes in labs where they were getting consistent results as per the contemporary standards. Furthermore, as the demand for better equipment rose steadily, new companies emerged in order to supply the growing ART lab industry with adequately designed disposables and equipment. It is apt to conclude that the single most important factor for the worldwide increase in IVF success rate over the last two decades was the commercial availability of purpose-designed devices and equipment for the IVF lab.

Even though medical/tissue culture laboratories embraced advanced automation workflow systems, the IVF laboratories still lag behind in introducing new technologies. Unlike any other medical or pathological laboratories, IVF labs need an error-free system as it deals with creation of human embryos.

There is a tendency towards a fully automated performance of the modern IVF unit targeting the sum of its functional aspects, from the assessment of the subfertile couple to the embryological procedures and medical interventions. In this context, several technologies have been proposed, and, although they are not yet fully established as reliable approaches in the clinical management of infertility, the interest is growing fast. As an example, Meseguer et al. recently described the automation of the IVF process and more specifically in sperm selection, oocyte manipulation and selection of culture media and embryos to be transferred [4]. It is generally accepted that, apart from the developing embryological technologies, prediction of IVF outcome would be the second most important achievement in assisted reproduction, behind a successful IVF cycle resulting in live birth. Numerous methods have been proposed since 1986 in an attempt to develop a functional system; more recent statistical methods and other newer techniques have contributed in a significant way ([5–7]).

Serving this purpose, specific artificial networks (ANNs) have been introduced during the last 30 years, representing a technological combination of a "learning", self-adapting and predicting system. Their application encompasses a number of functions, such as the ability to associate symptoms and disease, a "learning" path by cumulatively integrating specific data and the ability to foresee the exact treatment outcome. So far, such ANN systems have been utilized in gynaecological oncology, where early detection of a disease is a prerequisite, in reproductive endocrinology and assessment of the infertile couple with contradicting results, in urogynaecology with successful prediction of the outcome of the surgical procedures applied and in the early identification and management of menopausal and osteoporotic women [5]. In that paper, authors acknowledged the limitations and pitfalls in prediction precision of such systems, which arise from the incomplete and faulty "training" of the system during data input and the reported instability of some of them as predictive models per se, due to the association of the type of the preselected data used and their usually retrospective nature [5].

71.2 Current Developments in IVF Lab

71.2.1 Time-Lapse Embryo Culture

The incubator is one of the most important pieces of equipment in the IVF lab, because stable and accurate culture conditions are crucial for embryo development and IVF success. For almost two decades, the water-jacketed or dry heat-type box incubators were the centrepiece of equipment in any

ART lab: variations include (1) CO_2-only type, (2) tri-gas models, (3) mini box incubators to reduce the gas usage, (4) passive and active humidification models, (5) TC or IR sensor based, (6) different modes of sterilization options, (7) internal air cleaning filters, etc. However one of the major drawbacks of these incubators were the changes in internal gas phase and thereby pH variations due to frequent door openings. To avoid this, many labs started investing in multiple incubators, and still when we search the Google images, one can see IVF labs with rows of box incubators set against the wall. Even some labs used to dedicate one incubator for each patient. These practices consequently increase the space requirement and workload for maintenance. Bench-top incubators (Cook, K-systems, ESCO, Astec and Planer) function according to the "waffle iron" principle by providing close contact heating both from the top and from below directly on the culture dishes. The bench-top incubators, which are a quite recent evolution in IVF incubator technology, have been shown to improve IVF results through their rapid recovery times after lid opening (ref). Many IVF labs are now replacing their old CO_2 incubators with bench-top incubators in order to improve culture performance and reduce space.

Recently a self-contained incubation system integrated with time-lapse imaging has been introduced into IVF labs. Conventional methods of embryo scoring in most ART centres rely on the use of the inverted microscope, which offers information based on morphological and developmental characteristics from the early cleavage till the blastocyst stage. However, this method has several limitations [8, 9]. Notably, transfer of top-grade and morphologically good embryos often fails to result in clinical pregnancy, while embryos with poor scores sometimes result in live births. Apart from morphological assessment, invasive and non-invasive methods of selection have been developed. Single-cell biopsy at the cleavage stage has been reported to not interfere with embryo progression to the blastocyst stage [10]. In this context, preimplantation genetic screening (PGS) has been used for embryo selection and aneuploidy screening; however, its effectiveness has been placed under scrutiny [11]. Non-invasive techniques, such as embryo oxygen consumption, testing of soluble HLA-G, amino acid turnover, proteomics and metabolomics, cumulus cell gene expression analysis and time-lapse imaging (TLI), have currently become more popular options for embryo selection after IVF [12].

The rationale behind the use of TLI is based on the improved assessment of the embryos and their early cellular divisions, enabling to determine the timing of specific morphological occurrences and permit comparison between them while also identifying anomalies in the blastomeres and the nuclei, which otherwise would not be detected. This information can lead to more objective selection of embryos for transfer and/or cryopreservation, and the improvement of the success rates for ART patients, and for those diagnosed with repeated implantation failures [13]. Moreover, the potential undesirable shock or stress due to sudden changes in environmental parameters, such as temperature and pH associated with the use of a microscope, is avoided [14].

The technology is easily assimilated into the ART laboratory and is used with any culture medium and environment. Images of the embryos are recorded at regular intervals without their removal from the culture environment, and images can be viewed instantly or merged to form a video showing complete development from the oocyte to blastocyst stage: their review can assist in identifying and selecting embryos with normal developmental profiles and in deselecting those with abnormal phenotypes [15, 16].

Reports on the modality have been numerous during the last 10 years or more. The target was to explore the dynamics of an embryo to implant successfully along with its various cleavage patterns and characteristics, by observing it at various stages of development [17–20]. Recent studies reported information on the association of various confounding factors on embryo kinetics and development. Interestingly, the sensitivity and specificity of the modality in terms of predicting the progression to the blastocyst stage was >90% by measuring parameters at day 2 after fertilization, before embryonic genome activation [21], while the bad prognosis of good-performing but unviable embryos reaches a specificity of 100% [22]; moreover, the area under the receiver operating characteristic curve has been reported to be 0.74 for live birth [23]. In addition, a recent cohort found better reproductive outcomes, whereas the results were more evident in women aged more than 40 years, although there was no effect of TLM on outcomes, when clinical/external factors were taken under consideration; notably, authors observed more "in range" cellular events in certain embryo cycles in women aged 35–40 compared to those >40 years, in non-smokers than in smokers, in the GnRH-agonist group as compared to the GnRH-antagonist as well as in the embryos resulted in pregnancy compared to those that did not, indicating the importance of the timing of cleavage events on embryo competence [20]. The technology is an effective and safe alternative for embryo incubation, and no adverse effects have been observed in obstetric and perinatal outcomes when a time-lapse incubator was used compared to a conventional one [24].

On the other hand, there are contradicting views on the benefit of embryo selection using a time-lapse algorithm or computer-automated time-lapse image analysis test [25, 26].

According to meta-analyses conducted during the last 15 years, data show that there is insufficient evidence of differences in live birth, miscarriage, stillbirth or clinical pregnancy to choose between TLM and conventional incubation, so that recommendations for a change of routine practice cannot be justified and that the technique for embryo selection should remain experimental and that couples should not be subject to a surcharge for having their embryos cultured in it [27–30]. All of them highlight the low quality and limitations of the included studies. Furthermore, in the most recent RCT, the use of a TLI hierarchical classification model of day 3 single embryo transfer had a significantly lower ongoing pregnancy rate compared with day 5 SET with

conventional selection [31]. One recent study even shows that the manual annotation was superior to the automated annotation provided by Eeva™ version 2.2, because manual annotation assigned a rating to a higher proportion of embryos and yielded a greater sensitivity for blastocyst prediction than automated annotation [25].

However, in a validation study of the addition of automatic annotations and a classification algorithm related to reproductive outcomes, where logistic regression analysis studying confounding factors was included, authors reported that the automated embryo diagnostic test provided extra information to the embryologist to select the best embryos; if at least one of the embryos was labeled as high, ongoing pregnancy rate was 2.567 times higher than a cycle where no high embryos were transferred [32].

In a retrospective study [33], having a cohort size of 14,793 patients and 23,762 cycles, the incidence of live birth ($n - 973$ deliveries) after embryo selection by objective morphokinetic algorithms was compared with conventional embryology selection parameters ($n - 6948$ deliveries). A 19% increase in the incidence of live birth was observed when morphokinetic data were used to select embryos for the patient cohort aged younger than 38 years (OR 1.19 with 95% CI 1.06–1.34) using their own eggs and an increase of 37% for oocyte recipients aged over 37 years (OR 1.370; 95% Cl 0.763–2.450). This is the largest study of the prospective use of time-lapse imaging algorithms in IVF reporting on live birth outcome, although the nature of purely a closed system versus standard incubation could not be assessed in this study.

Even though there are contradicting reports on the pregnancy outcome with TLI, the system undoubtedly provides a consistent microenvironment for preimplantation embryo development in the extended culture. This definitely avoids the exposure of embryos to outer environment which is the norm in the conventional incubation systems. The future holds promise for TLI as the systems might get automated and the objective embryo selection will become a reality. Such systems already hold promise for integration into the artificial neural networking.

71.2.2 Novel Optical Systems for Laboratory

Among the microscopy systems being used in IVF laboratories, the stereo zoom microscopes are the workhorse of lab, and the inverted microscope caters to the micromanipulator, conventional embryo monitoring and scoring. Differential interference contrast (DIC) is the most popular imaging technique used for visualizing the living cells like spermatozoa in assisted reproductive technologies. The interference image by DIC has a quasi-3D appearance, where regions of differing optical thicknesses possess edges that stand out as either brighter or darker than their surroundings. Hoffman modulation contrast (HMC) is a phase contrast method that produces a pseudo-3D image similar in appearance to that of DIC but does so by employing a different optical mechanism. Intracytoplasmic morphologically selected sperm injection (IMSI) makes use of the DIC method to receive high detail, high contrast images of sperm cells. The DIC image is captured using a 60x or 100x oil objective and subsequently enhanced through the use of a magnification changer, video zoom and digital camera and imaging system. More than 10 years after the development of IMSI, the indications of the IMSI technique and its ability to increase pregnancy and/or live birth rates (compared with conventional ICSI) are still subject to debate. Results from the recent Cochrane review of RCTs do not support the clinical use of IMSI, and there is no evidence of effect on live birth or miscarriage, and the evidence that IMSI improves clinical pregnancy is of very low quality [34].

Interferometric phase microscopy (holographic microscopy) may be the next stage of advancement for imaging in ART. Holographic imaging provides the prospect to obtain quantitative images where images may possess meaningful values at all of their points. Holographic imaging generates quantitative data regarding the thickness of the cell and provides the clinician with data regarding the 3D structure of the cell. The sperm acrosome and nucleus can easily be identified and measured as in labelled sperm, as the acrosome has a significantly lower optical path delay (OPD) due to its lesser thickness. Vacuoles can be located and measured due to their decreased refractive index [35].

While current imaging methods have been used for decades, with the introduction of simple and portable holographic modules, holographic imaging is now poised to bring the field of sperm selection to its next stage of development.

In recent years spectroscopy including Raman spectroscopy has emerged as one of the major tools for biomedical applications and has made significant progress in the field of clinical evaluation. This Raman spectroscopic technique does not require sample labelling, getting biochemical information directly from the inelastic light scattering induced by its molecular vibrations. The Raman frequency shifts are conventionally measured in wavelength, depending on the atomic mass or molecular bonds of specimens; all the chemical information involved is presented on a Raman spectrum that can be further interpreted and analysed using statistical, chemical and morphological methods. Raman spectroscopy is a relatively simple, non-invasive and nondestructive technique providing molecular-level information, allowing investigation of functional groups, bonding types and molecular conformations [36]. Raman spectroscopy can be easily combined with complementary optical approaches, such as holographic microscopy.

Raman spectroscopy has been demonstrated to successfully detect sperm DNA damage, mitochondrial status and fertilization potential [37].

Researchers are also studying confocal Raman spectroscopy (CRS) which can provide information about oocyte competency through measurement of changes in the macromolecular architecture during oocyte development and maturation. Non-invasive Raman imaging of Metaphases I and II (MI, MII) and germinal vesicle-stage living oocytes showed

changes in nuclear organization and cytoplasm macromolecular architecture during these development and maturation stages related to changes in chromosome condensation, mitochondria aggregation and lipid droplet numbers [38].

Raman microspectroscopy has also been used to investigate vitrification-induced structural damages in mature bovine oocytes [39] and to analyse the oxidative stress-induced damages in mouse oocytes [40]. This non-invasive imaging could represent a highly informative method of investigation to evaluate the oocyte quality in the future.

Morphological features are considered the gold standard in embryo selection in IVF. However, the method seems to be empirical and it does not specify the embryonic quality. Therefore, new evaluation techniques for embryonic quality are highly desired. Near-infrared (NIR) and Raman spectroscopy metabolic markers of spent culture media have been reported to have correlation with pregnancy outcomes [41, 42]. However, according to the Cochrane analysis of current trials in women undergoing ART, there is no evidence to show that metabolomic assessment of embryos before implantation has any meaningful effect on rates of live birth, ongoing pregnancy, miscarriage, multiple pregnancy, ectopic pregnancy or foetal abnormalities [43]. Raman microscopy offers a more direct method to assess the embryonic quality, based on the molecular compositions obtained from the embryo non-destructively, non-invasively and without labelling.

Raman spectroscopy can provide rich molecular information non-destructively and in situ, without labelling the cells. Even in complicated biological systems like embryos, information about biomolecules such as proteins, lipids and DNA can be separately extracted from a Raman spectrum, and their quantitative variations and structural changes can be investigated. Ishigaki M et al. [44] measured in situ the Raman spectra obtained for unfertilized, pronuclear, 2-celled, 4-celled and 8-celled developmental stages. The variations in proteins and lipids and the structural changes in proteins concomitant with embryo development and morphology were observed non-destructively. Embryos with low-grade morphological feature have high concentrations of lipids and hydroxyapatite, indicating that the bands attributable to these components can be distinctive factors for the assessment of embryo quality. However the authors warn that it is essential to carefully examine the invasiveness of laser irradiation in embryos. This technique may be a novel and feasible method for non-invasive embryo selection based on molecular and biochemical markers.

71.2.3 Microfluidics in Assisted Reproduction

Conventional embryo production requires in vitro manipulation of gametes and embryos innumerable times which induces stress on the preimplantation embryos via external culture systems and microenvironment. Microfluidic systems can be employed to handle/process gametes, mature oocytes and culture embryos and perform other basic procedures in a microenvironment that more closely mimics in vivo conditions. It has been hypothesized that microfluidic environment may look more like the in vivo environment which is just opposed to the static system in an embryo culture Petri dish. Culture systems that are static do not permit real-time changes in the culture media that surround the embryo as it develops unlike the in vivo environment in which embryos grow in physiological fluids with a constantly changing environment. This changing environment likely occurs because some metabolites necessary during one phase of preimplantation development may not be needed during other stages. Since the microfluidic systems employ a confined and dynamic growth environment for embryos at sub-micro/nanolitre volumes, the autocrine and paracrine factors released by embryos can support better development. As the system is closed, there is no worry of media evaporation and thereby any osmolarity changes.

Microfluidic systems or lab-on-a-chip can perform multiple functions and manipulations: (1) motile and normal sperm isolation, (2) fertilization, (3) cumulus cell removal, (4) embryo culture and (5) embryo selection via metabolomics and time-lapse imaging. Since microfluidic devices are mostly made from optically transparent materials, they offer the possibility of embryo morphokinetic analysis. A combination of time-lapse imaging and oxygen-consumption assays was used to identify embryo selection before transfer [45], confirming its analytical potential.

Microfluidic platforms have also been explored for its utility in vitrification process. For instance, using digital microfluidics, Pyne et al. [46] accurately timed the exposure of a single mouse embryo placed in a sub-microliter droplet to various equilibration/vitrification solutions. They have shown that the process could be entirely automated, with similar results compared with manual procedures [46].

ICSI-on-a-chip microfluidic systems that combine the laboratory steps of ART by providing gamete preparation, selection and ICSI while reducing the use of hand labour have been reported by Palermo's team [47]. The system included a cast PDMS microfluidic chip with embedded piezo-driven injection system and valves with etched electrodes and wiring. Normal and motile sperm cells were isolated by laminar flow system, and cumulus denudation was carried out chemically and mechanically within dynamically created chambers and alternating suction channels. Embedded electrodes facilitated the orientation of the oocyte to allow maturity assessment. ICSI was performed on the mature eggs using a piezo-actuator-driven needle following trapping and immobilization of the sperm cell via laser. Oocytes were then transferred into individual embryo assessment chambers where fertilization and long-term embryo culture was monitored.

Automation of the microfluidic platforms is particularly essential for standardization of the processes in the IVF labs. An automated interface has been developed to pilot the entire in vitro culture of embryos in six independent chambers, with controlled medium delivery, possible changes in the medium composition and in situ regulation

of the temperature and gas tension [48]. The future holds many hypothetical promises with such systems. The IVF treatment could be fully customized and personalized, depending on the patient's profile like age, oocyte quality and physiological needs of the embryo based on individual patient needs. The use of integrated and fully automated platforms to perform the whole IVF procedure in an objective way would lead to standardization of the treatment across laboratories and subsequently increasing the take-home baby rates [4]. It is true to say that even after three decades of development and research, we are yet to see the clinical application of microfluidic systems in human IVF. Collaboration between automation engineers and biomedical and IVF scientists can pave way to circumvent many challenges posed in microfluidic research. However, new technologies need thorough validation using animal models, followed by donated human embryos and, finally, through randomized clinical trials before its widespread application in human IVF.

71.2.4 Automation in Vitrification

Cryopreservation of oocytes and embryos has become an essential component of ART procedures with more and more clinics opting for "freeze-all" policies. The last decade has witnessed several new vitrification media and devices along with multitude of hands on workshops in many congresses. However, due to poor reproducibility and inconsistency across operators, the success and survival rates also vary significantly. The embryo vitrification typically employs high concentrations of cryoprotectants followed by cryopreservation with liquid nitrogen to produce extremely rapid cooling rates which need to be performed with higher skill and time-bound manner. The survival rate and pregnancy rate vary with operators. To address the need for standardization of vitrification across users and clinics as well as a reduction in labour intensity, automated systems for vitrification hold much promise in ART lab.

One such semiautomated system, Gavi®, is designed with a robotic liquid handling unit that dispenses the necessary vitrification solutions, removing the need to manually move embryos between fluids. The Gavi system was developed at Genea (Sydney, Australia) and Planet Innovation (Melbourne, Australia). The Gavi system performs automated equilibration for closed system vitrification of up to four embryos simultaneously. The system is comprised of the instrument (Gavi) which performs fluid exchange using a robotic liquid-handling unit with individual pipettes, has a heat-sealing unit and includes the liquid nitrogen bucket, the single-use "pod" closed system device containing a novel microfluidic "channel" that secures the embryo during fluid exchange and vitrification. After equilibration, the pod is then heat-sealed, so that it can be placed directly into LN2, without any risk of contamination to the enclosed embryo [49]. However, the warming procedure needs to be done manually by the embryologist.

Yu Sun and team from University of Toronto is developing a robotic vitrification (RoboVitri) and ultimately aim to standardize clinical vitrification from manual operation to fully automated robotic operation. Their robotic system is embedded with two contact detection methods to determine the relative Z positions of the vitrification micropipette, embryo/oocytes and vitrification straw. The system is integrated into an inverted microscopic stage. A 3D tracking algorithm is developed to visually observe the embryo loading and real-time monitoring of embryo volume changes during vitrification. Excess medium is automatically removed from around the vitrified embryo on the vitrification device to achieve a desired high cooling rate. Tests on mouse embryos demonstrate that the system is capable of performing vitrification with a throughput at least three times that of manual operation and achieved a high survival rate (88.9%) and development rate (93.8%) [50].

The capability of automated pick and place of single embryos enabled the robotic system to perform vitrification of multiple embryos in an optimally scheduled sequence. The system is claimed to pick and transfer embryos into equilibration solution by automated visual inspection and again transferring them from ES to vitrification solution. The system was able to process six embryos in 24 min. Only failure in RoboVitri arose when an embryo floated in the VS solution and happened to drift into blind regions of the multiwell plate.

Amir Arav has developed a fully automated device – SARAH – which claims to have (1) automatic stepwise exposure to equilibration and vitrification solutions, (2) automatic cooling into LN or LN slush, (3) automatic warming by exposure to warming solution and (4) automatic stepwise dilutions and washings. The device is claimed to vitrify/warm simultaneously up to 30 embryos/oocytes (mini straws) and up to 18 ovarian slices (maxi straws).

The automatic device to vitrify oocytes and embryos consists of a vertical robotic handle where a special straw holder, which can load up to six straws, is attached. This robotic arm moves in a vertical plane (up and down), at predetermined time intervals, and by so doing carries the biological samples contained in the straws between different solutions (vitrification and equilibration) arranged into nine cups placed in a temperature-controlled metal carousel plate. The final station on the carousel plate is the one containing LN where the straws are ultimately plunged and the entire cycle of vitrification is considered completed.

Prior to attaching the straws to the holder, the oocytes or embryos are first manually loaded into the straws, and then the straws are closed at one extremity by special capsules (50 μm pores) (Fertile Safe Ltd., Nes Ziona, Israel). After the holder is placed on the robotic handle and the protocol has been selected from the touchscreen, the process begins by touching the "ON" command. Once the entire preparatory steps are completed, the holder plunges the straws into a special insulated vessel containing LN or sterile liquid air. The special straw holder is then disconnected from the handle, and the straws can be either stored "as it is" or they can be

inserted into a 0.3 ml straw heat-sealed and then placed in LN tanks for long-term storage.

After the 100% VS and prior to immersion into LN, there is a step where the straws are inserted into a cup containing an absorbing paper to remove the VS and achieve the lowest volume possible.

The warming procedure consisted of first plunging the straws into 100% warming solution (WS) at 37 °C for 5 s and then immediately placing the straws in the vertical robotic arm of the SARAH device. In a reverse process than used for vitrification, for the rewarming the straws are automatically plunged into temperature-controlled 5 ml tubes containing warming solutions kept at room temperature, before arrival into the final holding medium station.

Arav A et al. [51] reported the preliminary successful results using the SARAH automated vitrification system with mice and bovine oocytes and embryos. Rewarmed 8-cell mice embryos had 95% (33/35) blastulation rate and 80% (28/35) hatched. Fresh control mice embryos had 100% (42/42) blastulation and 73% (21/42) hatching rates. Vitrified bovine oocytes had 100% survival (84/84), 73% (61/84) cleavage and 7% (6/84) blastocysts rates; fresh control had 83% (125/150) cleavage and 11% (17/150) blastocysts rates.

Samuel S. Kim et al. describe an automated device comprising a cryoprotectant holder, a cryoprotectant dispenser and a sample holder oriented to allow a sample to be in contact with cryoprotectant from the cryoprotectant dispenser. In this device, oocytes or embryos are kept in a sample holder (e.g. porous mesh) throughout the entire procedure, while the vitri/warming solutions are dispensed in and drained out of the device. The sample holder is a rotating sample holder that moves the sample from a position that allows introduction of the sample to a position that allows introduction of the cryoprotectant from the dispenser. The rotating sample holder further moves the sample from the position that allows introduction of the cryoprotectant to a position that allows sealing of the sample by the sample sealing device. The order, time and duration of administering the vitrification/warming solutions to the biological samples are said to be controlled via a control module.

In the very near future, we will witness different automated/robotic systems for cryopreservation getting into the day-to-day practice of embryologists. Many of them are still in prototype development or in the research/clinical trial phases. The major question posed by the inventors of automated devices is: How to equilibrate the embryos? This can be done by limited ways only. In manual human interventions, the embryos are moved from solutions to solutions by visual control. The same logic is applied by Sun et al. in their RoboVitri system where the system picks and drops the embryos in the desired area/solutions. In the second option, one has to park the embryo in a vessel and drop and drain the desired solutions in and out of the vessel. This is applied in the Gavi system. However, the optimal minimum volume of final vitrification solution around the embryos is in question. The third option is park the embryos in a device/vessel with porous embodiment. The desired fluids pass through the device/vessel while equilibrating the embryos with the necessary cryoprotectants.

The survival and quality of warmed embryos not only depend on the standardization of vitrification process but also on the warming procedure. Hence I'm of the opinion that the automation must look into both the process of vitrification and warming. Since there are many commercially available vitrification/warming media being used by laboratories, the system should cater to different brands. This may be achieved by software programming. Moreover the designs should integrate vitrification – warming and storage of the samples in the same system. The smart chip (Bluechiip, Australia) is a passive device based on microelectromechanical systems (MEMS) technology that contains no electronics and is either embedded or manufactured into cryogenic storage equipment such as vials, cassettes, straws, plates, racks and towers. Such sensors have the potential to get integrated into the automated storage vessels to track and retrieve cryosamples at desired time points.

71.2.5 IVF Workstations

IVF workstations have been evolved across the years from the simple horizontal/vertical laminar flow stations to dedicated flow hoods with warm benches and to isolator-type closed benches. IVF hoods provide the safe environment of all gamete/embryo manipulations. Even some versions contained dedicated area for integration of ICSI workstation. The ideas behind all these developments were to provide a physiological niche for gamete/embryo manipulation without much variation in physical parameters like pH, temperature, humidity, etc. Very recently a spurge of developments has been observed in many systems used in ART lab which fortunately have been done with collaboration with embryologist and inputs from scientists working in ART.

Comparison of conventional hood and enclosed chamber for all the IVF process reported that significantly more embryos developed to the blastocyst stage in the enclosed isolator-based system compared with conventional open-fronted laminar flow hoods [52].

Even though studies show the better physiological outcomes with enclosed workstations, they are yet to become popular among laboratories. Embryologists prefer to work with open workstations as it gives more convenience for movements. The open workstations are amenable to modifications which can yield an integrated work area cum incubation locus. This will also avoid embryologists running between workstations and incubators. These incubators integrated in hood can be either drawer type or time-lapse imaging incubators. A 6 feet workstation can also integrate on one side an advanced monitor only (no-ocular)-based inverted microscopy system with an incubation chamber on microscope stage for culture dish observation. This enclosed observation will help embryo scoring at physiological microenvironment. Moreover an integration of morphology scoring software in touchscreen monitor with standard

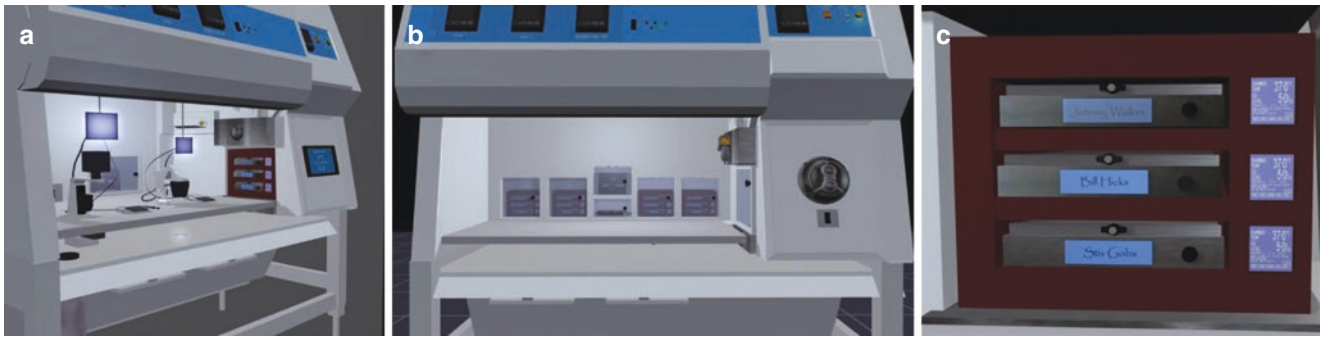

Fig. 71.1 A proposed model of an integrated open IVF workstation with ocular lens-digital microscopes **a** with image/data processing and multiple voice command drawer-type incubators **b**, **c**. The model also integrates electronic witnessing system along with a cloud-based QC app. (image provided by Alex C Varghese)

morphology patterns will make the process easy for embryologists (Fig. 71.1). Clinical embryology-specific microscopes need to be developed for this purpose. The size of culture media drop harbouring the oocytes/embryos is smaller with regard to the magnifications of inverted microscope objectives. This makes it difficult to locate/focus the embryos across the droplets. The operator needs to go back to lower magnification (5x) to see the droplet position and then shift to the higher objective to get a clear view of the embryos. This process during the embryo scoring also causes delay and thereby shifts in pH. Automation is feasible during the scanning of different droplets of media harbouring embryos. This along with an incubation chamber for the embryo scoring will make a portable and economically cheaper embryo scoring microscopy system.

Another area amenable to automation and ensured error-free process is a station to prepare and incubate the required number/type of dishes for each day/time as per the laboratory requirement. A prototype model is being developed (by the first author's team) with robotic fluid handling systems and software programming which may be helpful for busy laboratories and will ease the weekend duties of lab personal.

71.2.6 Artificial Intelligence

Subfertility is currently characterized as a disease with variable socioeconomic extends. Up to date, the IVF success rates remain relatively low, ranging from 4% to 40% based on various parameters, mainly patients' age. We estimate that this is due to an inaccurate cumulative assessment of couples' reproductive characteristics, the IVF cycle itself and the lack of incorporating indications from important biological aspects, such as molecular investigations and "omics". In this respect, improvement of IVF outcome would be a unique achievement in assisted reproduction and systems, such as computerized algorithms, hybrid architectures and ANNs are exceptional candidates in providing the fertility specialist with numerical estimates to promote personalization of healthcare and adaptation of the course of treatment according to the indications [6].

The rationale of implementing artificial intelligence in ART procedures, either clinical or embryological, is to enhance the probability of offering to infertile couples the best consultation/options for a successful outcome after IVF. Such a system can be scheduled to act as a routine information technology platform for the everyday IVF unit, either alone or in many of them. Its capability is mainly based on recalling and evaluating a vast amount of information, previously selected and usually in a retrospective, while rapid and automated, manner, which is used to provide an objective indication on the outcome of an artificial reproductive cycle. Among functions that its application involves, it is its ability to associate symptoms and disease, a "learning" path by cumulatively integrating specific data, and to foresee treatment outcome, the most important ones. Such systems have been utilized in a variety of clinical situations. Examples include in gynaecological oncology (where early detection of a disease is one of the ultimate goals), in reproductive endocrinology (where it is used for the assessment of an infertile couple with contradicting results), in urogynaecology (in successfully and early predicting both symptoms and outcome of surgical procedures), in the early identification and management of menopausal and osteoporotic women and in obstetrics in the intrapartum foetal monitoring [5, 6, 53–55].

On the other hand, an ANN has flaws, which are related to the objectivity of the network expert and medical staff responsible for the system "training" and the types of input parameters or the input of "true" or resolved cases during training. For this, careful interpretation of the dynamics of such system is of major essence. The obtained results should be in a harmonic balance with the clinical judgement and decision-making themselves, the latter to constitute the main part of clinical management that could not be substituted by any form of artificial intelligence, at least in its present form. In the same context, it is essential for obtaining the right output/result from the system, to evolve and readapt it, continuously; this can be achieved through incorporating new technologies and new feedback based on various IVF units experience that use it. This can be tested through a robust statistical evaluation of the degree of its predictive power in order to be adopted in an optimal way and establish its dynamics.

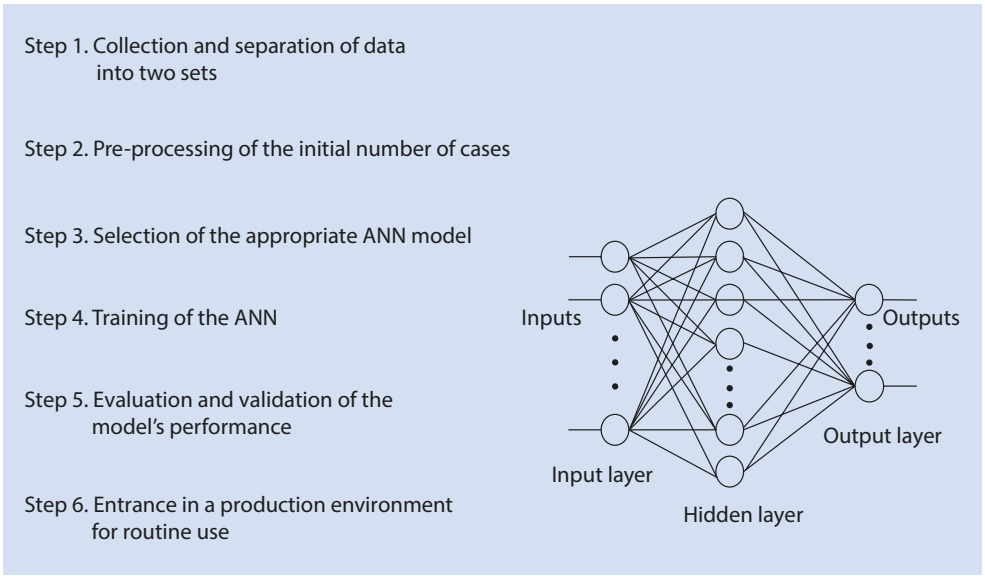

Fig. 71.2 Architecture of an ANN and steps of production

71.3 Description of an ANN

Historically, ANNs were first described in 1943 when McCulloch and Pitts popularized the concept of the artificial neuron: they reported that, by combining artificial neurons, mathematics and algorithms, they could create an artificial network similar to a biological one [56]. In 1949, Hebb introduced the concept of Hebbian or associative learning [57], where a learning mechanism could be applied in excited cells, to get information of their reaction. The idea of training came in 1962 from Frank Rosenblatt, who suggested that synaptic strengths had to be altered each time the network gave a wrong answer, while Werbos introduced the idea of propagating the errors back to former layers of the network ("back propagation of the error algorithm") [57]. Likewise, Hopfield et al. described that an asynchronous network could adjust and find a minimum, using the "least energy", so that problems requiring minimization of the network output could be solved, evolving a more biological reaction and identity [58].

The construction of an ANN is linked with the application of specialized algorithms addressing specific issues, varying from the investigation of couple's subfertility to those related to the organization and the performance of the IVF laboratory. The difference between these systems and the conventional computational ones is their ability to "learning through training". The function of an ANN resembles to "the capacity of the brain to learn and subsequently assimilate and recall this knowledge in anticipation of a future prospect; through supervised training, the characteristics of the structural components of the ANN are adapted and change in order to store the acquired knowledge" [6]; in this respect, the base of such a system is consisted of training algorithms that modify the characteristics of its components and the information flow through the network neurons [57].

The construction and the final function of an ANN consist of several steps/basic training principles [5, 6, 55] (Fig. 71.2):

1. The first step includes the collection and the separation of data into two sets, namely, the training and the test sets (at a 50/50 division), whereas percentages up to 80% for training sets have been reported [59]. The size of the data set depends on the nature of the question set a priori. Importantly, this size – usually – is changed upwards following evaluation of the primary results, so that if they are not satisfactory, new data are inserted in the initial data set, reprocessed, also clearing inconsistent entries and entries with missing data. Interestingly, an ANN trained by using all data cannot be considered as satisfactory for the addressed problem, because it cannot be guaranteed for usage on unknown data. For instance, ANN models can be trained to achieve very high performance on the training set, but eventually to exhibit poor performance in the test set (a problem known as "overtraining").
2. The second step includes the preprocessing of the initial number of cases and depends on the ANN type and the parameters selected. This is achieved by clearing any inconsistent values and by performing mathematical transformations suitable for the selected algorithm of the ANN model. The final goal is double: (a) to select the appropriate characteristics for the ANN training and to create the definitive input data set and (b) to separate these data into, at least, two sets, the training set and the test set, which are used to evaluate its training and performance, respectively [57, 59].
3. The third step is related to the selection of the appropriate ANN model and the definition of the characteristics of its components (neurons), while, according to the model, further individual characteristics are adjusted in advance.

4. The fourth step includes the training of the ANN, which is accomplished via the exposure of the training data set to the first network layer and by the fine-tuning of the system parameters. Importantly, a large number of cases with a previously known outcome are necessary for this purpose [59]. In specific, the training involves repeated steps of parameterization, evaluation of outcomes in the training set, extraction of performance metrics and validation on the test set (and/or the combined data set).
5. The final step comprises evaluation and validation of the model's performance, through the use either of the test set or the complete data set. Specificity and sensitivity are estimated, and ANN is tested if it is valid and has acceptable performance. The target in this step is the presence of an acceptable performance for the training set and the existence of no differences with the test set. This shows that the model parameters are fine-tuned. Further validity is checked by splitting the data several times via stratified random sampling, and the use of other models retraining through the same parameters found. In contrast, if it is not, all steps are re-evaluated from the beginning. Notably, a threshold of more than 75% for both positive and negative predictive values is considered to be a satisfactory result for the specific addressed problem.
6. A further step encompasses its entrance in a production environment for routine usage.

The ANN relies on the use and evolution of the particular set of algorithms that are implicated. The learning algorithms and the whole process are prone to pitfalls. Interestingly, most of them are encountered during the training stage [59]. The majority of them include the definition of the ANN dimensionality; the selection of the appropriate parameters; the inability of most ANNs to be trained again at any stage, when, for example, new data are at a later stage; the fact that most ANNs demonstrate poor performance at the test set, although it is excellent during the training set, because they are trapped to mathematical local minima; and the usual requirement of excessive computing power during the training stage [5, 6].

Current ANNs incorporate many and very popular algorithms, such as the back propagation algorithm-based networks; radial basis function-based networks; networks with recurrent architectures, such as Hopfield-type networks; systems with self-organization, such as the popular learning vector quantization network and the self-organizing feature map, both targeting clustering problems; and systems based on Hebbian learning, as referenced in two reviews of Siristatidis 2010 and 2011 [5, 6].

Technically, the justification/reasoning procedure is based on a mechanism that identifies the cases of the training set that have the greatest similarity to an unknown case; thus, the class of the unknown case is similar to the class of the cases used during training. Various types of ANNs have different characteristics, rendering their choice crucial before their use, for example, characteristics that are of great importance are its fast convergence; its resistance when getting trapped in data-related local minima, thus minimizing stability problems; and its generalization capability, which represents its performance on unknown data. Robustness of the ANN model is achieved with the random splitting of the data for several times, creating and training again new models using the same parameters, expecting that their performance on the new training and test sets are similar [57, 58].

Such models can be used in various combinations with the new web-based technology. As an example, a web-based multi-unit ANN model was proposed: the architecture involved the construction, evaluation, production and utilization of the model, for the everyday needs of the embryology lab; its main components included the server farm (consisting of the database server, the web engine and the application server), prediction models (with well-defined inputs and outputs available as web services), databases and, finally, the IVF unit, the lab and the patients [55]. Importantly, a further important key factor is the quality of the data inserted, for example, data of the metabolomic profile of the embryo and transcriptomics of the oocyte and endometrium.

A simple scenario of use could embody several poses (Fig. 71.3): the insertion of personal data into the system database, such as data from demographic and medical history of partners, clinical examination and previous IVF cycle and laboratory characteristics; call the artificial intelligence service supplied by the platform in order to evaluate the couple's probability for a successful outcome; response of the system with several alternative treatment scenarios; consultation of the physician by this service; clinical decision with the minimum risk of failure or complications; storage of all IVF cycle-related data in the database; the ANN supplied by the artificial intelligence service is used to evaluate the success rate and to provide risk details for the next step of the cycle; and all recorded results are again used for the training and test of the ANN model, thus enhancing its predictive potential [55].

Such models are expected to receive a wide acceptance as an innovative managerial tool in fertility centres worldwide and could possibly be established in routine clinical practice for the evaluation and management of infertile patients in the near future. The upper ambition is to end up to a personalized model used by the patient herself, improve the cost-effectiveness of an IVF cycle and provide a more targeted approach with more accurate predictions. This will eventually prevent the patient from potentially unnecessary costly tests or treatments, especially in cases with poor prognosis (e.g. advanced age, poor ovarian response) and provide more efficient resource exploitation. Additionally, the identification of a single best quality embryo for transfer promotes the reduction in multiple gestation rates and therefore potential complications, again reducing overall expenses for national health systems. The beneficial impact by alleviating the emotional state of the couple, through minimizing stress related to the IVF procedures and the anticipation for a positive outcome and the systematic adoption of intelligent systems with "omics" in IVF clinics could prevent extensive delays in family planning caused by multiple IVF attempts resulting in

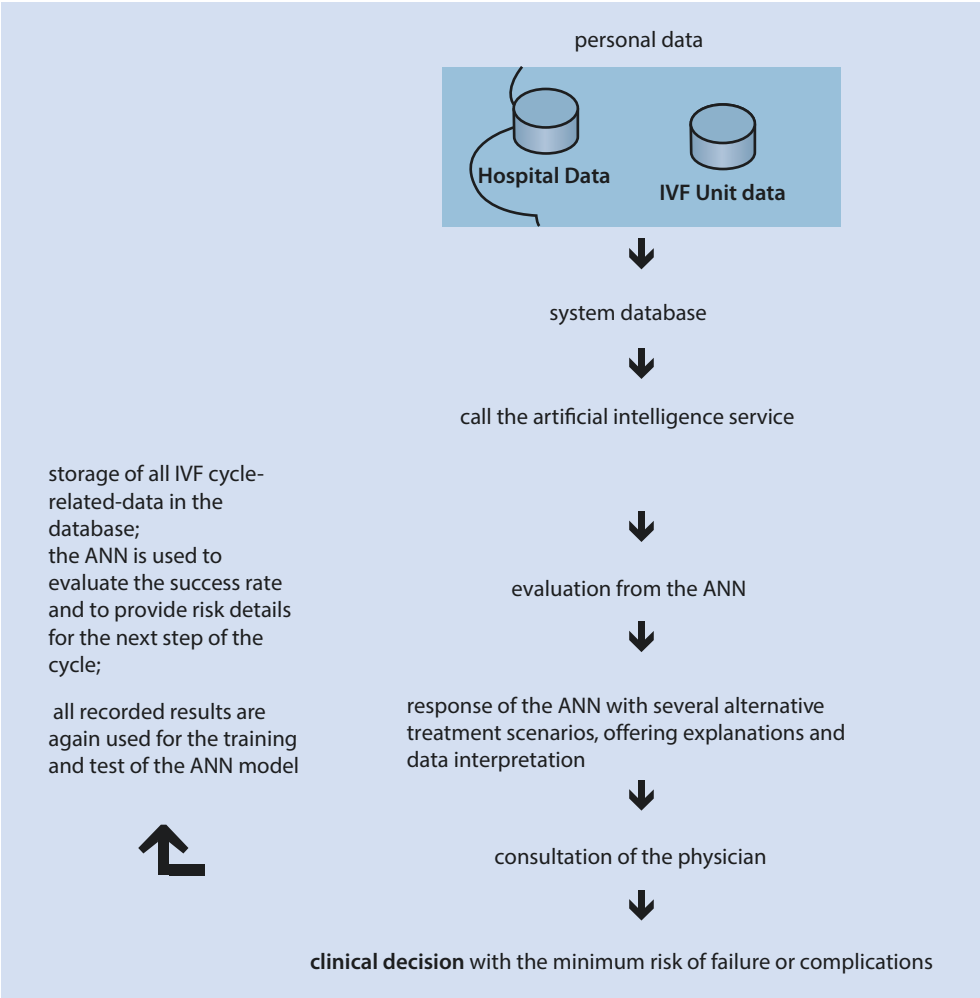

Fig. 71.3 The flow from personal data to clinical decision using an ANN

delayed parenthood and its accompanying consequences. From an ethical viewpoint, this intervention allows the minimization of cycles with poor prognosis, cycles with no embryo transfer and embryo biopsy due to failed IVF cycles (e.g. recurrent implantation failure) resulting from disregarding other potential indications leading to negative outcome.

71.4 A Typical Example on the Use of Artificial Intelligence in Embryo Selection (Life Whisperer: An Industry-Specific Use Case)

Life Whisperer uses computer vision and deep-learning artificial intelligence (AI) to assist the selection of healthy embryos in IVF and to ultimately improve outcomes for couples wanting to have children. An international clinical study involving 12 IVF centers across the USA, Australia, New Zealand and Malaysia has demonstrated that Life Whisperer's AI performs 30% better than highly skilled embryologists when identifying viable embryos from medical images – a critical pregnancy success factor for couples undergoing IVF.

Life Whisperer's embryo viability assessment model (Fig. 71.4) was created by developing a set of image recognition algorithms, which are then trained on a large data set of embryo images with matched outcomes. The output of this process is a classification model that can effectively assess and identify morphological features that constitute a viable embryo. Features detected by the model are of two kinds: targeted features previously established in the scientific literature (e.g. size, shape or texture) or more complex features and patterns that arise from performing deep learning on an extensive data set. The computer vision-AI approach can identify combinations of features in the images, some of which are impossible to see with the human eye and difficult to identify consistently using a manual approach.

Life Whisperer is delivered to IVF clinics globally via a scalable and easily accessible cloud/web-based app, which allows embryologists to drag and drop images of patient embryos immediately prior to implantation. The embryologist then receives an instant report as to the viability of each embryo. Embryologists can then use this report to support their decision on which embryo to implant. Life Whisperer's web-based approach does not require any costly hardware and thus can be delivered at a low cost to patients. This

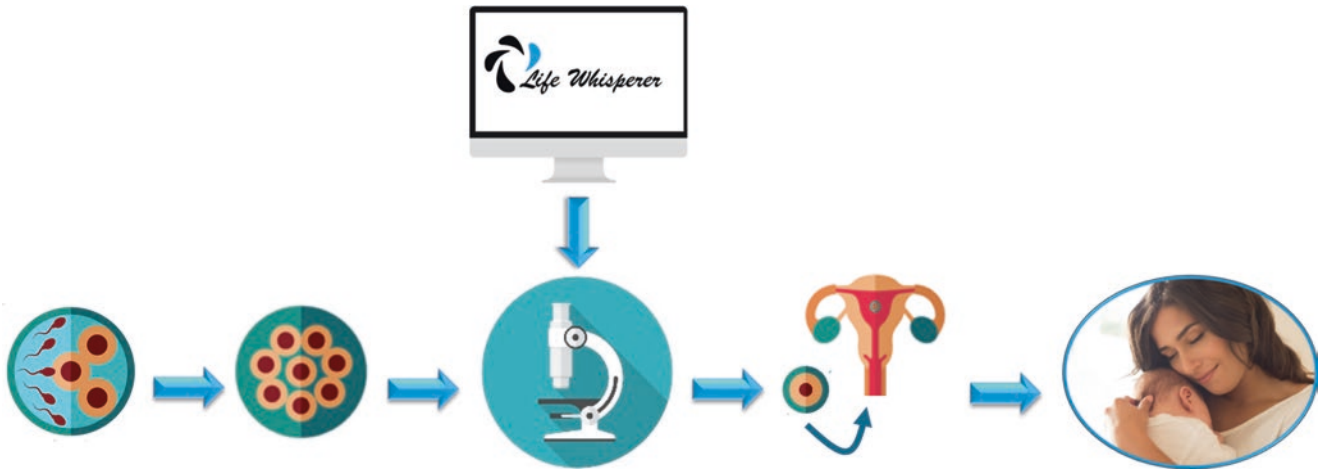

Fig. 71.4 Overview of the IVF process and the application of Life Whisperer web-based embryo viability assessment tool. (We acknowledge Life Whisperer Diagnostics Pty Ltd., Australia, for the figure and information about the Life Whisperer AI system)

approach also incurs limited change of process for the embryologists carrying out IVF.

The IVF process starts with an ovarian stimulation phase which stimulates egg production. Eggs are then retrieved from the patient and fertilized in vitro with sperm. Embryos develop over a period of up to 5 days, after which time they are expected to have formed a blastocyst suitable for transfer back into the patient. The selection of the best embryo at the point of transfer is critical to ensure a positive pregnancy outcome. Then an embryologist visually assesses the embryos using a microscope to make this selection. Many clinics already record images of the embryos at the point of selection. The embryologist then scores each embryo based on various metrics and their visual assessment down the microscope. The selected embryo is then transferred back to the patient.

71.5 Conclusion

The assisted reproduction technologies (ART) labs provide resources for all steps of IVF treatment: gamete handling, IVF or micromanipulation, embryo culture, preparation for embryo transfer and cryopreservation. There is a tendency towards a fully automated performance of the modern IVF unit targeting the sum of its functional aspects: from the assessment of the subfertile couple to the embryological procedures and medical interventions.

Time-lapse imaging, as a system of an improved automated way of observing the quality of embryos, provides a consistent microenvironment for preimplantation embryo development in the extended culture. This avoids the exposure of embryos to outer environment, which is the norm in the conventional incubation systems. The future holds promise for such automated systems, while the objective embryo selection will become a reality. Also, they can be integrated into the artificial neural networking.

Special imaging techniques are currently being used in the IVF laboratory, including micromanipulators, conventional embryo monitoring and scoring and differential interference contrast techniques, used for visualizing the living cells. Microfluidic systems have been employed to handle/process gametes, mature oocytes and culture embryos and perform other basic procedures in a microenvironment that more closely mimic in vivo conditions. From the freezing techniques, cryopreservation of oocytes and embryos through vitrification has become an essential component of ART procedures with more and more clinics opting for "freeze-all" policies. IVF workstations have been evolved across the years from the simple horizontal/vertical laminar flow stations to dedicated flow hoods with warm benches and to isolator-type closed benches. IVF hoods have been integrated to provide a safer environment of all gamete/embryo manipulations.

Improvement of IVF outcome would be a unique achievement in assisted reproduction; systems, such as computerized algorithms, hybrid architectures and networks based on artificial intelligence (ANNs) which are exceptional candidates in providing the fertility specialist with numerical estimates to promote personalization of healthcare and adaptation of the course of treatment according to the indications. The construction of an ANN is linked with the application of specialized algorithms addressing specific issues, varying from the investigation of couple's subfertility to those related to the organization and the performance of the IVF laboratory. The difference between these systems and the conventional computational ones is their ability to "learning through training". The function of an ANN resembles to "the capacity of the brain to learn and subsequently assimilate and recall this knowledge in anticipation of a future prospect; through supervised training, the characteristics of the structural components of the ANN are adapted and changed in order to store the acquired knowledge". Such models can be used in various combinations with the new web-based technology. They are expected to receive a wide acceptance as an innovative managerial tool in fertility centres worldwide and could possibly be established in routine clinical practice for

the evaluation and management of infertile patients in the near future. The upper ambition is to end up to a personalized model used by the patient herself, improve the cost-effectiveness of an IVF cycle and provide a more targeted approach with more accurate predictions. This will eventually prevent the patient from potentially unnecessary costly tests or treatments, especially in cases with poor prognosis, and provide more efficient resource exploitation.

Review Questions

1. Is the result of an IVF cycle today satisfactory?
2. Why has IVF not reached 100% in terms of positive outcomes?
3. Why do IVF labs need error-free systems?
4. Which are the scientific areas where ANN systems have been utilized?
5. What is the difference between time-lapse imaging and conventional methods for observing the embryos?
6. How is a more objective selection of embryos for transfer and/or cryopreservation linked with the improvement of the success rates for ART patients?
7. Which are the contradicting views on the benefit of embryo selection using a time-lapse algorithm or computer-automated time-lapse image analysis test?
8. Which are the applications of Novel Optical systems in a modern IVF laboratory?
9. What is the rationale of using microfluidic systems in the IVF laboratory?
10. What are the advantages of vitrification as a cryopreservation modality?
11. Why can automated vitrification be linked with the improvement of the success rates for ART patients?
12. What is the rationale of using IVF workstations?
13. Why are computerized algorithms, hybrid architectures and ANNs candidates in the improvement of the success rates for ART patients?
14. What are the steps/basic training principles for the construction and the final function of an ANN?
15. Give examples on how ANNs models can be used in various combinations with the new web-based technology.

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Assisted Reproductive Technologies to Prevent Transmission of Mitochondrial DNA Disease

Louise Hyslop

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Learning Objectives

- Reproductive options for women with mitochondrial DNA disease
- Advantages of vitrifying the patient rather than donor oocytes in preparation for the pronuclear transfer procedure
- Methodologies for minimizing carryover of mtDNA during PNT

Mitochondria are double membrane-bound cytoplasmic organelles located in the vast majority of cells of all eukaryotic organisms. Oocytes contain mainly spherical- to oval-shaped mitochondria, but during preimplantation embryo development, the morphology changes to a bean-like structure (see [1] for review of mitochondrial morphodynamics in human oocytes and embryos). Mitochondria have many functions, but the main role is in production of adenosine triphosphate (ATP) which is a source of chemical energy.

Mitochondria contain their own genome which is a circular DNA molecule (mtDNA) containing only 37 genes that encode 13 proteins, 22 transfer RNAs and 2 ribosomal RNAs. The mitochondrial gene-encoded proteins have a key role in energy production. Mitochondria are classified into haplogroups according to similarities or differences in the mtDNA sequence. mtDNA is maternally inherited with fertilized oocytes containing more than 200,000 copies. If all the copies of mtDNA have a mutation, this is known as homoplasy, whereas if the mutation is present in only some of the copies of mtDNA, this is known as heteroplasmy and the mutation load is the ratio of mutated to wild-type mtDNA. Pathogenic mtDNA mutations above a certain threshold of mutation load can cause a range of mitochondrial diseases. The severity of disease is determined by the mutation load with high risk of severe and life-threatening diseases with high mutation loads [2]. The transmission of mtDNA disease is unpredictable because women with a heteroplasmic mtDNA mutation can produce oocytes with varying mtDNA mutation loads.

72.1 Assisted Reproductive Options for Women with mtDNA Disease

72.1.1 PGD

PGD for mtDNA disease can reduce the risk of transmitting mtDNA disease and is an option available for women with a heteroplasmic mtDNA mutation. The mutation load of the biopsy sample is determined, and embryos with low mutation loads can be selected for transfer. Extensive analysis of a range of pathogenic mutations indicates the probability of developing mtDNA disease is low when the mutation load is less than 18% [2].

72.1.2 Oocyte Donation

Until the development of mitochondrial donation techniques, oocyte donation was the only option for women with a homoplasmic mutation or who only produce oocytes with a high mutation load.

72.1.3 Mitochondrial Donation

Mitochondrial donation has been developed for women with homoplasmic mutation or who consistently produce embryos with mutation levels above the threshold for mtDNA disease. The strategies involve transplantation of the nuclear DNA of either an oocyte or fertilized oocyte from an affected woman to that of a donor oocyte which has had its nuclear DNA removed.

1. Pronuclear transfer (PNT) – transplantation of the pronuclei in a fertilized oocyte from an affected woman to that of a donor-fertilized oocyte which has had its pronuclei removed (Fig. 72.1). Each pronucleus is transferred in a membrane intact karyoplast and fused with the enucleated zygote known as the cytoplast.
2. Maternal spindle transfer (MST) – transplantation of the spindle in an oocyte from an affected woman to that of a donor oocyte which has had its spindle removed (Fig. 72.2). The spindle is transferred in a membrane-intact karyoplast and fused with the enucleated oocyte known as the cytoplast.

These techniques could enable women to have a genetically related child with a greatly reduced risk of transmitting mtDNA disease. The risk is not eliminated since some mtDNA is carried over during the transplantation of the nuclear DNA.

72.2 Safety and Efficacy of Mitochondrial Donation

72.2.1 Carryover of mtDNA

The level of mtDNA carryover is low with both MST and PNT in studies with human oocytes and fertilized oocytes [3–5]. Various groups have derived embryonic stem cell lines from MST and PNT generated blastocysts to explore the fate of the carried over mtDNA after extended culture. The majority of human embryo stem cell lines had a similarly low level of karyoplast-derived mtDNA as the blastocysts from which the lines were derived. However there have been exceptions reported with an increased proportion of karyoplast-derived mtDNA in the ES cells and a wide variation between colonies compared to the blastocysts from which the cells were derived. It has been suggested that differences in mtDNA replication between haplotypes could explain why the carried over karyoplast-derived mtDNA is preferentially amplified in

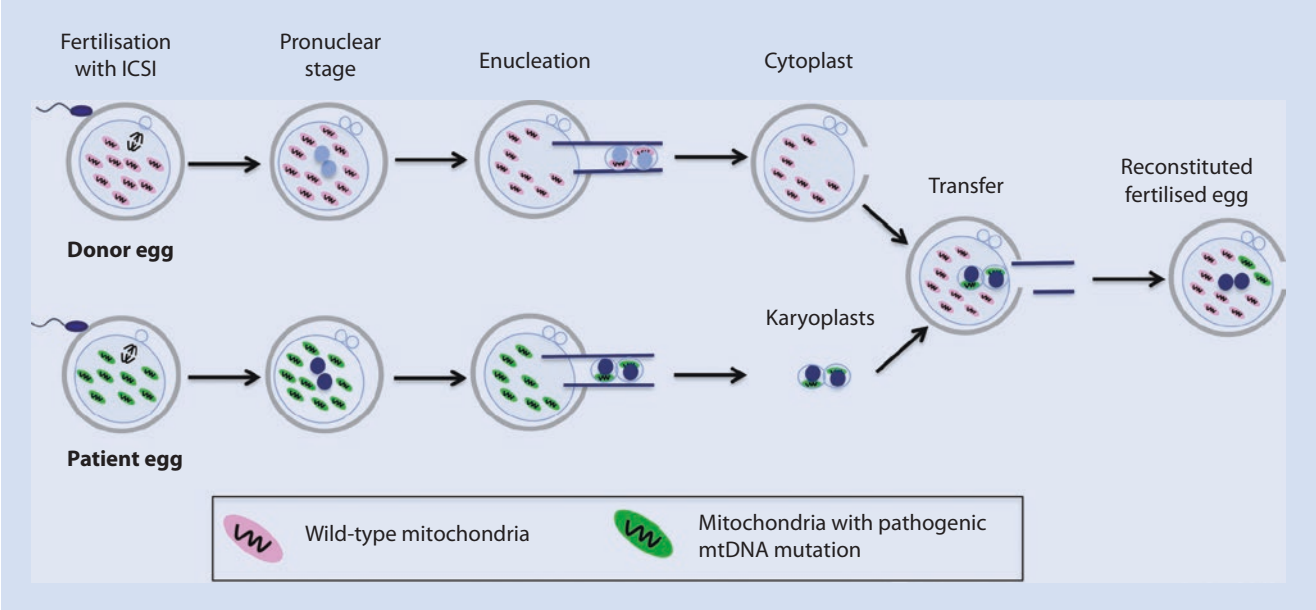

Fig. 72.1 Schematic of pronuclear transfer. Pronuclei are removed from the patient- and donor-fertilized oocytes within intact karyoplasts. The karyoplasts containing the patient's pronuclei are fused with the donor cytoplast. The reconstituted embryo contains mainly mitochondria from the donor-fertilized oocyte, but some carried over mitochondria contained in the patient's karyoplasts

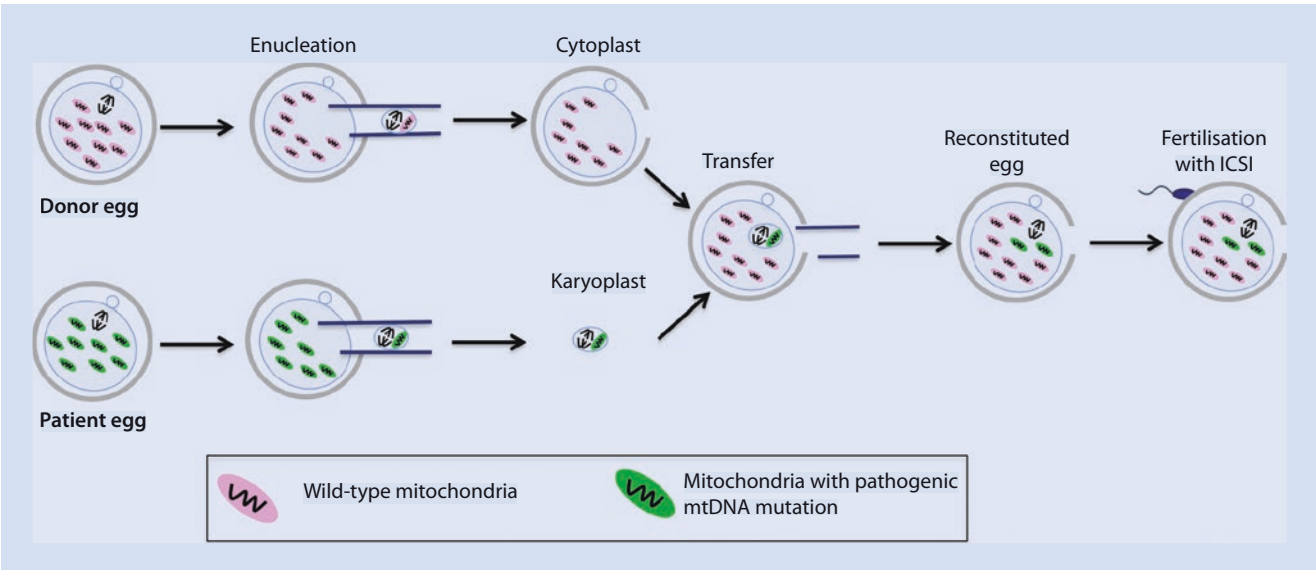

Fig. 72.2 Schematic of maternal spindle transfer. Spindles are removed from the patient and donor oocytes. The karyoplast containing the patient's spindle is fused with the donor cytoplast. The reconstituted oocyte contains mainly mitochondria from the donor oocyte, but some carried over mitochondria contained in the patient's karyoplast

the ES cells after prolonged culture. For this reason the haplogroups of the patient and egg donor should be recorded for the long-term follow-up of any children born as a result of treatment. Another possible reason for the upward drift is variation in sequences that interact with the mtDNA replication machinery. mtDNA replication is not fully understood, so further research is required to elucidate potential reasons for the upward drift. It should be noted that the relevance to what might happen in utero is unclear, so further research is required and long-term follow-up of births.

72.2.2 Embryo Development Post-procedure

Following MST the fertilization rate has been reported to be similar to controls, but there was a high incidence of abnormal fertilization with 25% of fertilized MST oocytes containing three pronuclei with a single polar body [5]. Studies are required to compare the blastocyst morphology and quality to unmanipulated controls in order to provide an indication of efficacy.

The blastocyst development rate of PNT zygotes was improved by performing the procedure shortly after appearance of the pronuclei rather than just prior to syngamy. Analysis of control and PNT blastocysts has revealed the PNT procedure had no detectable effect on gene expression and the incidence of aneuploidy.

72.2.3 Clinical Treatment

There are mixed views on the replacement of female embryos generated using mitochondrial replacement techniques. A report from the National Academies of Sciences, Engineering, and Medicine recommended only the transfer of male embryos to prevent carryover of mutated mtDNA to subsequent generations until there is clear evidence of safety and efficacy [6], whereas the expert panel convened by the HFEA to perform the fourth scientific review of the safety and efficacy of mitochondrial donation does not support the transfer of only male embryos [7]. Determining the embryo sex would require biopsy of the manipulated embryos for PGD which could further compromise embryo development and reduces the cohort of embryos from which to select the best quality for transfer.

At time of publication, a live birth had been reported following MST, and pregnancies were ongoing following PNT [8, 9]. MST was performed for a woman with a heteroplasmic mtDNA mutation. The mutation load of the embryo replaced was 5.7%, and the baby had a mutation load range of undetectable to 9.23% in different tissues at birth. PNT has been performed for couples that consistently produced poor-quality embryos rather than to reduce the risk of transmitting mtDNA disease. Therefore the carryover of mtDNA is not known for the embryos replaced following PNT.

72.3 Regulation of Mitochondrial Donation

In 2015 the UK was the first country to put legislation and a licencing framework in place to allow the use of mitochondrial donation. Centres in the UK can offer mitochondrial donation once a licence variation application has been assessed and approved by the HFEA's License Committee. Areas assessed include the process for following up children born as a result of the treatment and competence of the embryologist in performing the technique and minimizing carryover. Subsequently if mitochondrial donation is identified as a suitable treatment for a couple, the clinic must send a patient-specific application to the HFEA for assessment by the Statutory Approvals Committee. The evidence must demonstrate that without mitochondrial donation, there would be a significant risk that a child will have or develop serious mitochondrial disease. For example, a woman may have had PGD for mtDNA disease but consistently produced embryos with high mutation levels.

72.4 Pronuclear Transfer

72.4.1 Technical Aspects of Pronuclear Transfer

The key stages of the pronuclear transfer procedure include (a) creation of a hole in the zona to facilitate the insertion of the biopsy pipette, (b) pinching off each pronucleus with a minimal amount of cytoplasm to form a membrane-enclosed karyoplast and (c) fusion of the karyoplasts with an enucleated zygote (cytoplast).

72.4.2 Timing

Early pronuclear transfer soon after the appearance of the pronuclei maximizes the timeframe for the karyoplasts to fuse and pronuclei centralize prior to syngamy.

72.4.3 Laser-Assisted Opening of the Zona Pellucida and Enucleation

To facilitate the enucleation procedure, the zygotes are transferred to medium containing reversible microtubule and actin-depolymerizing drugs. Creating the hole in the zona pellucida should be performed as quickly as possible to minimize the exposure of the zygote to the inhibitors. The zygote is positioned with both pronuclei in focus and closer to the biopsy pipette than the holding pipette (Fig. 72.3). The size of the opening created in the zona pellucida should be just sufficient to allow easy insertion of the biopsy pipette and will assist with the pinching off of excess cytoplasm during the

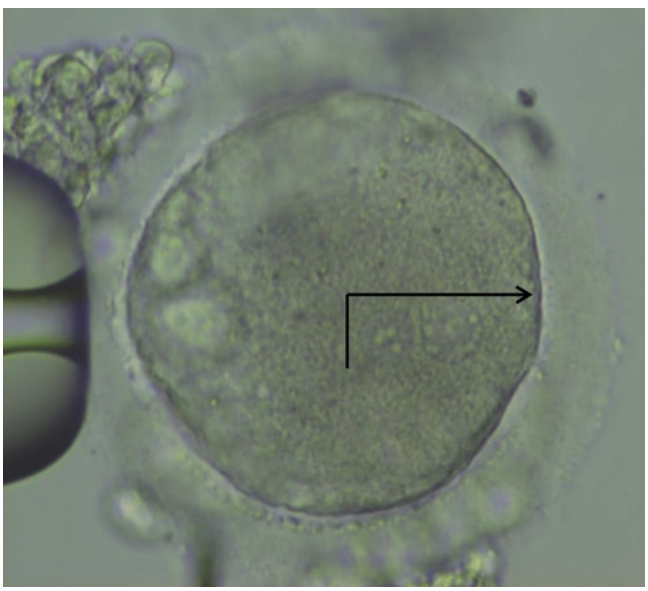

Fig. 72.3 Normally fertilized oocyte positioned prior to removal of the pronuclei. Both pronuclei are in focus and closer to the biopsy pipette that will be inserted at the 3 o'clock position than the holding pipette on the left-hand side

transfer. Too large an opening increases the risk of karyoplast expulsion prior to fusion.

The biopsy pipette is inserted and positioned to ensure there is minimal cytoplasm between the pronucleus to be aspirated and the biopsy pipette. The pronucleus is gently and slowly aspirated into the biopsy pipette. If too much cytoplasm is aspirated before the pronucleus, the biopsy pipette should be repositioned to minimize carryover. Once the pronucleus starts to enter the biopsy pipette, the pipette is slowly removed to facilitate separation. The karyoplast is expelled from the biopsy pipette and the procedure repeated for the second pronucleus.

72.4.4 Fusion

The most efficient method of fusion of the karyoplast and enucleated zygote membranes is with the fusion reagent hemagglutinating virus of Japan envelope (HVJ-E). Both karyoplasts are transferred in the biopsy pipette to a drop of the HVJ-E solution. It is critical that the karyoplasts are not expelled into the HVJ-E solution because the likelihood of lysis during replacement increases. Expel the karyoplasts until the leading karyoplast reaches the opening of the biopsy pipette. Aspirate the karyoplasts to ensure the leading karyoplast is moved away from the opening of the biopsy pipette and only a small amount of HVJ-E is aspirated into the tip of the pipette. Only the karyoplast closest to the opening of the pipette is exposed to the HVJ-E, but this is sufficient to achieve efficient fusion of both karyoplasts with the cytoplast. Transfer the biopsy pipette to the drop containing cytoplast. Using the holding pipette, immobilize the cytoplast with the hole in the zona positioned at 3 o'clock. Insert the biopsy pipette and slowly expel the karyoplasts beneath the zona pellucida (Fig. 72.4). Any excess cytoplasm in the karyoplast can be removed by pinching the karyoplast against the zona pellucida using the tip of the biopsy pipette. The risk of expulsion of a karyoplast prior to fusion is reduced if the karyoplasts are not deposited just underneath the hole in the zona pellucida. The risk is further minimized by the use of a large embryo manipulation pipette for washing the reconstituted zygote in embryo culture medium before transfer to the final dish for overnight culture.

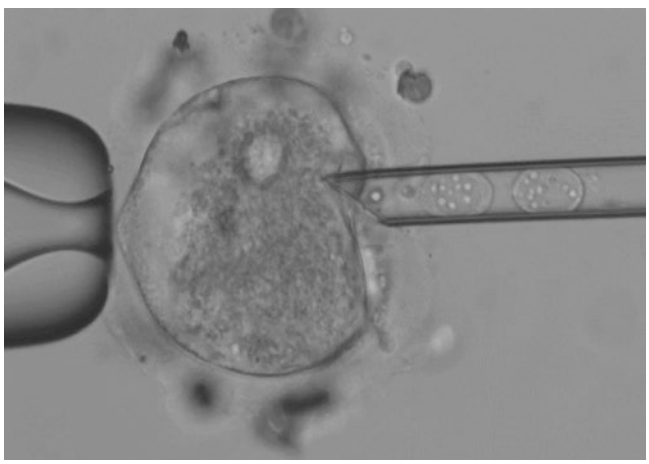

Fig. 72.4 Karyoplasts in the biopsy pipette prior to being deposited in the perivitelline space. The biopsy pipette has been inserted through the hole in the zona pellucida to allow the karyoplasts to be deposited

72.4.5 Training

During the training period, the trainee's competence in minimizing mtDNA carryover should be assessed alongside survival and onward development of the embryos. A realistic expectation is a new operator at the end of the training period will achieve approximately 90% survival and more than 95% fusion. The trainee should perform technical controls by removing and replacing the pronuclei in the same zygote (autologous transfer). It has been shown that the blastocyst development rate and blastocyst quality should not differ from unmanipulated controls [3]. In the UK, evidence of experience and competence is assessed by the HFEA. Only those assessed as competent are named on the clinics HFEA licence and are able to perform MST and/or PNT.

72.4.6 Practical Considerations for PNT

72.4.6.1 Synchronization of Patient and Donor Cycles

Synchronization of the patient and donor cycles for oocyte retrieval on the same day can be challenging. Vitrification of the patient rather than the donor oocytes has a number of advantages including:

- Banking of patient oocytes before they become susceptible to maternal age-related aneuploidy.
- To optimize usage of the patient oocytes, the number to be warmed can be based on the final follicle count of the donor. This would minimize the likelihood of not having enough donor-fertilized oocytes compared to the number of fertilized patient oocytes created for the procedure.
- Preclinical studies demonstrated that the carryover of mitochondria was lower if the cytoplast originated from the fresh rather than vitrified oocyte. Vitrification of the patient oocytes may minimize carryover.

72.4.6.2 Method of Insemination

ICSI synchronizes the PN appearance in a narrower timeframe than IVF insemination. This increases the likelihood of being able to perform the procedure with zygotes at a similar stage post appearance of the pronuclei.

72.4.6.3 Pronuclei Removal to Minimize Carryover

To minimize carryover, the pronuclei should be removed separately and not together in the same karyoplast. When early pronuclear transfer is performed, the pronuclei are rarely abutted so removal of both pronuclei in the same karyoplast will carry over cytoplasm between the pronuclei.

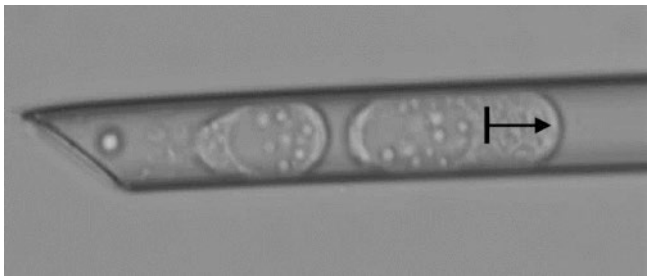

Fig. 72.5 Positioning of the excess cytoplasm in the biopsy pipette prior to transfer into the perivitelline space. Arrow indicates the excess cytoplasm to be pinched off during transfer of the karyoplasts

72.4.6.4 Transfer of Pronuclei to Reduce Carryover

If a karyoplast contains excess cytoplasm, the karyoplast should be aspirated into the pipette first by the excess cytoplasm before the pronucleus (Fig. 72.5). During the transfer the excess cytoplasm can be sheared with the tip of the biopsy pipette against the zona pellucida.

72.5 Maternal Spindle Transfer

72.5.1 Technical Aspects of Maternal Spindle Transfer

The key stages of the MST procedure are similar to PNT except the spindle is removed within a karyoplast. Polarization microscopy is required to visualize the meiotic spindle. HVJ-E is preferred to electrofusion for fusion of the cytoplast and karyoplast because of the higher rate of embryo viability and the lower rate of activation of the manipulated oocyte without fertilization [4, 10].

72.5.2 Timing

The timing of spindle recovery has been reported to be faster in vitrified oocytes compared to slow frozen [11, 12]. It is recommended that the optimal timing to visualize the maternal spindle should be determined within each centre for fresh and frozen oocytes.

72.5.3 Laser-Assisted Opening of the Zona Pellucida and Enucleation

For polarization microscopy, the oocyte manipulation dish needs to be a glass bottom dish. To facilitate the enucleation procedure, the oocytes are transferred to the manipulation dish with drops of medium containing a reversible actin-depolymerizing drug. Creating the hole in the zona pellucida should be performed as quickly as possible to minimize the exposure of the oocyte to the inhibitor. The oocyte is positioned with the spindle in focus and located between the 1 and 3 o'clock position. The size of the opening created in the zona pellucida should be just sufficient to allow easy insertion of the biopsy pipette and located next to the spindle.

The biopsy pipette is inserted and positioned to ensure there is minimal cytoplasm between the spindle to be aspirated and the biopsy pipette. The spindle is gently and slowly aspirated into the biopsy pipette with a minimal amount of cytoplasm. Once the spindle starts to enter the biopsy pipette, the pipette is slowly removed to facilitate separation. The karyoplast is expelled from the biopsy pipette and the procedure is repeated.

72.5.4 Fusion

The karyoplast containing the spindle is transferred in the biopsy pipette to a drop of the HVJ-E solution. It is critical that the karyoplast is not expelled into the HVJ-E solution because the likelihood of lysis during replacement increases. Expel the karyoplast until it reaches the opening of the biopsy pipette. Aspirate the karyoplast to ensure it has moved away from the opening of the biopsy pipette and only a small amount of HVJ-E is aspirated into the tip of the pipette. Transfer the biopsy pipette to the drop containing cytoplast. Using the holding pipette, immobilize the cytoplast with the hole in the zona for replacement of the karyoplast at 3 o'clock. Insert the biopsy pipette and slowly expel the karyoplast beneath the zona pellucida. The reconstituted oocyte is transferred to a culture dish to allow fusion of the karyoplast and cytoplast. After confirmation of fusion and spindle recovery, the oocyte should be fertilized by ICSI.

72.5.5 Training

During the training period, the trainee's competence in minimizing mtDNA carryover should be assessed alongside survival and normal fertilization rate of the reconstituted oocyte. A realistic expectation is a new operator at the end of the training period will achieve more than 90% fusion of the cytoplast and karyoplast containing the spindle.

72.5.6 Practical Considerations for MST

The issue of synchronizing the patient and donor cycles for egg collection on the same day have been described earlier in the chapter. The time difference between warming the oocytes and the fresh oocyte retrieval should be carefully considered based on local protocols.

72.6 Future Direction for Mitochondrial Donation Techniques

A current concern is the upward drift in the karyoplast-derived mtDNA in the minority of ES cell lines with prolonged culture. Methods have been refined to minimize the amount of cytoplasm transferred within the karyoplast so alternative techniques are being investigated to reduce further or eliminate the carryover of mtDNA. Polar body transfer is

one example of a methodology which could further reduce the carryover of mtDNA. Polar bodies contain a very low number of mitochondria and are ready-made karyoplasts for fusion with an enucleated donor oocyte. This methodology has shown promise when a study using human oocytes demonstrated that following polar body transfer the levels of aneuploidy were similar between control and polar body-derived blastocysts.

Review Questions

1. What are the current reproductive options for women with mtDNA disease?
2. Why is pronuclear transfer performed shortly after appearance of the pronuclei?
3. Is it recommended that the patient or donor oocytes can be frozen prior to performing the mitochondrial donation techniques? What are the advantages?
4. What KPIs should be assessed to determine the competence of an embryologist training in MST and PNT?

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Nuclear and Cytoplasmic Transfer: Human Applications and Concerns

Josef Fulka Jr and Helena Fulka

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Learning Objectives

- Chromatin modifications during oocyte maturation
- Basic equipment necessary for nuclear or cytoplasmic transfer
- Problems that can be solved with nuclear and cytoplasmic transfer
- Future implications and concerns of nuclear and cytoplasmic transfers

73.1 Characteristics of Mammalian Oocytes

Mammalian oocytes are fascinating cells, and their relative big size makes them very convenient for different manipulations. In laboratory animals and also in ungulates, they can be obtained easily in sufficient number (e.g., from the slaughterhouse material). On the other hand, human oocytes are very rare. In humans, the collected oocytes are almost exclusively used for the production of test tube embryos and babies, and only minimum of IVF centers use spare oocytes for different micromanipulations, aiming to find and improve some approaches that can be used for some specific patients. Unfortunately, not all oocytes collected are morphologically and physiologically normal. When working with laboratory animals or ungulates and oocytes from a given collection is not good, we can simply discard them saying that the next collection will certainly be better. Logically, the situation in humans is completely different, and it is not so easy to discard those oocytes that are not absolutely perfect, especially in those patients where this situation is repeatedly observed. Thus, in such difficult situations, the micromanipulation approaches might represent a solution. However, if we want to manipulate abnormal human oocytes, we need also some normal oocytes from healthy donors. Another important aspect is that we still do not know if babies from manipulated oocytes will be absolutely normal as long-term follow-up studies are often not available. Because some very recent articles discussed the manipulations of mammalian oocytes and one-cell stage embryos in detail in our contribution, we will outline briefly, from a general point of view, the micromanipulation possibilities that can be applied almost immediately in human-assisted reproduction [1, 2].

73.2 General Morphology

The size of mammalian oocytes ranges from about 80 (mouse) to 120 μm (human, cattle, pig), so they are relatively large. But even in this case, they cannot be simply manipulated by hand. Therefore, good equipment is absolutely essential. In human-assisted reproduction, the oocytes are almost exclusively collected as mature, i.e., in metaphase II stage, when they can be used directly for fertilization. However, in general, the oocytes can be collected at different stages of maturation: either as immature with intact nucleus—germinal vesicle (GV), maturing with condensed chromosomes (metaphase I)—or mature (metaphase II). The choice of maturation stage for further manipulation clearly depends on the problem that needs to be addressed.

Immature oocytes contain the GV (nucleus) that is easily visible in rodent or human oocytes. The most prominent organelle that is visible in GVs is the nucleolus. In fully mature competent oocytes, the nucleolus is surrounded with a ring of chromatin (SN—surrounded nucleolus). On the other hand, in oocytes that are less or even not competent to mature, the chromatin is dispersed within the GV (NSN—nonsurrounded nucleolus) [3]. The oocytes are enclosed with zona pellucida and several layers of cumulus cells.

After an endogenous (exogenous) gonadotropin surge or when released from follicles and cultured under appropriate conditions, the oocytes begin to mature. Their nuclear envelope disassembles (germinal vesicle breakdown—GVBD); chromosomes condense and become gradually arranged in metaphase I. These processes are followed by a very short anaphase to telophase I transition, and the oocytes are thereafter arrested in metaphase II [4]. Two essential points must be mentioned here. First, the oocytes matured in vitro are not as good as those oocytes where this process underwent in vivo; therefore, in vitro matured oocytes usually have a lower developmental potential. Second, if we want to manipulate immature oocytes, we must remove their surrounding cumulus cells. This will even further decrease their developmental potential after fertilization [5]. However, when we need to solve, for example, the problem of absolute oocyte maturation arrest, i.e., the oocytes are unable to undergo GVBD, and we suspect that it is the cytoplasm that is responsible for this arrest, there is no other choice than to use these immature oocytes.

On the other hand, if oocytes are unable to undergo metaphase I to telophase I transition or when we suspect that their cytoplasm influences abnormal segregation of chromosomes (leading to aneuploidies), we can collect these oocytes from follicles at this stage (MI or shortly before). This later collection might be beneficial because the period of GVBD is essential for an establishment of oocyte developmental competence, and we may suppose that these oocytes are as good as those oocytes maturing completely in follicles. Logically, from the oocyte developmental competence point of view, best for manipulations are MII oocytes that matured completely in vivo.

73.3 Oocyte Maturation: Chromatin Modifications

As outlined above, the timing of oocyte collection might influence negatively their developmental potential. Although this might be caused by a variety of different factors and the effect of oocyte collection timing is extremely complex, it has been convincingly shown that the oocyte collection timing might influence the epigenetic status of oocytes.

The process of oocyte maturation is very dynamic from many aspects (cell cycle, distribution of organelles, etc.). This is certainly also true for different chromatin modifications.

Even though the epigenetic regulation of chromatin is very complex, in this section, we will focus mainly on histone acetylation as there is growing evidence indicating that the acetylation/deacetylation processes are in a close correlation with oocyte aneuploidies. In general, when probed for acetylation at different lysine residues within histones (H3/K9, H4/K12, H4/K5, etc.), it is evident that histones are highly acetylated in GV-stage oocytes. However, as soon as GVBD occurs and chromosomes condense, the labeling signal disappears, indicating that the process of chromosome condensation is associated with histone deacetylation. During the anaphase to telophase I transition, a weak signal on chromosomes can be observed again, but at metaphase II, no labeling can be detected [6, 7]. Interestingly, if the acetylation persisted during the process of oocyte maturation, an increased number of oocytes (embryos) had chromosomal abnormalities [8]. It is also apparent that aged oocyte chromatin becomes also gradually aberrantly acetylated, and in agreement with this, oocytes from older females tend to exhibit more aneuploidies than oocytes from younger females. It is commonly accepted that it is the abnormal oocyte cytoplasm that is unable to deacetylate the condensing chromatin, indicating that histone deacetylases (HDACs) are either present in insufficient quantities or are aberrantly regulated. In contrast to histone acetylation, the histone methylation pattern remains rather constant during the whole process of maturation [9]. As we mentioned above, these results were mostly obtained in the mouse, and thus, some minor differences can be expected when analyzing the oocytes from other species. To summarize, a link between the cytoplasm quality and/or oocyte collection scheme and epigenetic status of oocytes has been established; in turn, the epigenetic competence of oocytes might influence the frequency of aneuploidies. Thus, the micromanipulation techniques might solve the problem of poor oocyte cytoplasm quality; however, care should be taken when performing the oocyte collection, and especially a correct timing is necessary.

73.4 Technical Aspects

73.4.1 Necessary Equipment

Some very recent articles describe in detail the necessary equipment, media, steps, and settings of manipulation chambers when different oocyte or embryo manipulations are performed. For this reason, we will not discuss here exhaustively everything what is necessary. Essentially, every IVF lab performing the ICSI has the very basic equipment necessary for nuclear or cytoplasmic transfer—this means the inverted microscope with manipulators and injectors and, logically, also some stereomicroscopes and incubators. To perform more sophisticated manipulations, some additional instruments are however necessary. According to our opinion, the most important is the electrofusion machine because the diameter of nuclear material (either GVs or chromosomes) is rather large, and it cannot be simply injected into the host cytoplast without damage. Thus, in this case, the induced fusion between the nuclear material (karyoplast) and recipient cytoplast (enucleated oocyte) is recommended. For the visualization of chromosome groups, the PolScope optics seems to be very useful. The holding pipettes can be purchased from different companies. The injection pipettes can also be purchased, but it is our experience that it is much better if they are made directly by the person performing the manipulation. In this case, the pipette puller and microforge are necessary [1]. We strongly recommend some training with mouse or ungulate oocytes. Moreover, it is absolutely essential to study some manuals dealing with basic oocyte and embryo culture procedures and manipulations [10].

73.4.2 Brief Outline of Manipulation Methods

In general, and as mentioned above, the various manipulation schemes do not differ substantially from ICSI. What is however different, if we want to manipulate GVs or metaphase groups, is the composition of manipulation medium that must contain cytochalasin B (or D) to avoid the damage of biological material. However, the CB (D) is omitted from fusion media. Again, some excellent protocols describe in detail what to do and how to perform different manipulations [1, 11–13]. Logically, these protocols must be slightly modified depending on the origin of cells used and the type of manipulation. Essentially, this however only means to modify the diameter of injection pipette and possibly the fusion parameters.

73.5 Problems That Can Be Solved with Nuclear and Cytoplasmic Transfer

73.5.1 Possible Nuclear Transfer Combinations

In theory, the manipulation of mammalian oocytes can solve some serious problems, i.e., the inability of oocytes to begin to mature, metaphase I or metaphase II arrests, or the inability of oocytes to transform the injected sperm head into a paternal pronucleus [14]. In all those cases, we can expect that the oocyte cytoplasm is abnormal, and this means to transfer the nuclear material from an abnormal cytoplasm into a normal cytoplasm from which its own nuclear material has been removed previously (cytoplast). It is also speculated that the transfer of nuclear material from the cytoplasm which is unable to induce histone deacetylation will eliminate the possible abnormal chromosome segregation, leading to prevention of aneuploidies.

It is technically relatively simple to remove the nuclear material from evidently morphologically abnormal oocytes and to transfer it into the cytoplasm that is normal. Although technically simple, this procedure might bring some serious concerns. The most often discussed method is the transfer of

nuclear material from oocytes with mutated mitochondrial DNA (mtDNA) into the cytoplasts with normal mtDNA. In the next section, we will discuss the possible combinations of karyoplast-cytoplast transfer. Clearly, the choice of the combination used in the micromanipulation scheme largely depends on the problem that needs to be solved [15].

73.5.2 Immature Oocytes: GV Transfer

In assisted human reproduction, the oocytes are mostly collected as mature—metaphase II staged—where they can be used immediately for fertilization. However, mammalian oocytes can be collected from follicles as immature (GV staged) and matured in vitro. The same is true for human oocytes. The main disadvantage is that the quality of in vitro matured oocytes is much lower when compared to oocytes matured in follicles. If we want to manipulate immature oocytes, we must free them from enclosing cumulus cells. This will further decrease their developmental potential after fertilization. It must be noted that in almost all cases mentioned below, the nuclear material is transferred into a recipient cytoplasm in the form of the so-called karyoplast. This means that the isolated nucleus (chromosomes) is enclosed with a minimum volume of original cytoplasm enclosed with the plasma membrane. The oocytes without the nuclear material are called "cytoplasts."

Applications:

- Elimination of mutated mtDNA. This means that the GV is isolated from the cytoplasm containing mutated mtDNA and transferred under the zona pellucida of another oocyte that was enucleated previously and contains normal mtDNA. The introduction of GVs into cytoplasts is typically induced by electrofusion. The reconstructed oocytes are then cultured in vitro until they reach metaphase II stage when they are fertilized. The question that remains is how to eliminate the residual karyoplast mitochondria.
- Maturation arrest. Exceptionally, the collected oocytes are immature even after hCG stimulation, and they do not undergo GVBD in culture. We may suppose that their cytoplasm is defected and unable to produce some essential cell cycle regulation factor. In theory, this problem can be solved by transferring GVs from oocytes with defected cytoplasm into cytoplasm (cytoplasts) obtained by enucleation of normal oocytes. The reconstructed oocytes will be then matured in vitro. Possibly, the isolated GVs could be transferred into more maturation-advanced cytoplasts, i.e., obtained by enucleation of oocytes undergoing GVBD but before the exit from MI. Under the influence of cytoplast's chromosome condensation activity (CCA), GVBD will be induced, and chromosomes will condense and subsequently reach MII stage. However, it must be noted that transfer of less developmentally competent GVs with chromatin nonsurrounded nucleoli (NSN) will not increase developmental competence of reconstructed oocytes.
- Elimination of aneuploidies. The percentage of aneuploidies increases with the age of patients. Thus, it has been originally suggested that transfer of GVs from "old" oocytes into "young" oocyte cytoplasm will eliminate this problem. Experiments in the mouse however did not support this presumption.
- Repairing evidently defected oocytes. The gross morphology of some collected oocytes is sometimes evidently abnormal (e.g., their cytoplasm is not homogenous). Logically, even if these oocytes are able to mature, we cannot expect that further embryonic development will be normal. Theoretically, healthy-looking oocytes can be produced by GV transfer.

Above, we have mentioned some possible applications of GV transfer. Technically, and of course with some experience, this approach is rather simple. GVs can be located in the cytoplasm very easily without special optics (mouse, human). Moreover, GV karyoplasts can be efficiently stored in liquid nitrogen and used later on if, for example, the recipient cytoplasts are not available at the same time [16]. The main disadvantage is the fragility of immature oocytes. However, the reconstructed oocytes mature well in culture, and even some offspring were obtained in the mouse. At the same time, we must bear in mind that the period of GVBD seems to be very important for very early postfertilization steps—e.g., it has been shown to be important for the demethylation of paternal DNA—and it remains to be determined how and to what extent the embryonic development and normality of offspring will be influenced if GV transfer schemes are used to produce offspring.

73.5.3 Maturing Oocytes

The oocytes undergoing GVBD and not yet achieving the metaphase II stage are designated as "maturing." Saying simply, these oocytes can be either at prometaphase, metaphase I, anaphase I, or telophase I. Anaphase and telophase I stages are rather short, and for this reason, their manipulations will not be discussed here. The maturing oocytes can be obtained either from stimulated follicles (i.e., in humans after approximately 24 h post-hCG) or possibly when oocytes are cultured in vitro, again after approximately 24 h postinitiation of culture. Because in this case the oocytes underwent GVBD in follicles, their quality is much higher when compared to completely in vitro matured oocytes and is essentially the same when compared to completely in vivo matured oocytes. Another important point is that the absence of cumulus cells that must be removed prior to manipulation has a minimum influence on the final stages of oocyte maturation. Compared to immature oocytes, the maturing oocytes are refractory to damage. The main disadvantage is a very poor visibility of chromosome groups. This problem can be overcome, for example, when oocytes are stained with some vital DNA stains (Hoechst) and then irradiated with UV light. In human-assisted reproduction, we recommend the use of

PolScope optics enabling the visualization of spindles without UV.

Applications:

- In theory, the elimination of abnormal segregation of chromosomes during the anaphase to telophase I transition due to the inability of the original oocyte cytoplasm to deacetylate histones
- To overcome the possible metaphase I arrest

It is our opinion that this approach will be used only exceptionally (i.e., to overcome metaphase I arrest). Its further use and possible justification need additional studies not only in laboratory animals and ungulates. For example, it is critical to analyze the histone deacetylation processes in human oocytes and if the proper deacetylation can be indeed induced by transferring the chromosomes into appropriate cytoplasts.

73.5.4 Mature Oocytes

As mentioned above, human oocytes are mostly collected as mature, i.e., metaphase II staged. Logically, their maturation in follicles secures their best quality, although we cannot exclude that an abnormal follicular environment (hormone levels) especially in older patients is responsible for a compromised oocyte quality and the incidence of oocyte aneuploidies. As for MI, the mature oocytes (MII) are refractory to damage. Essentially, the manipulation of these oocytes is very similar to MI oocyte manipulation.

Applications:

- Elimination of mutated mtDNA by isolating metaphase II chromosome group from the cytoplasm with mutated mtDNA and its transfer into cytoplasts with normal mtDNA. This can be either done by electrofusion of karyoplasts with cytoplasts or by direct injection of a spindle with chromosomes into the cytoplast. The spindle isolation and reinjection, however, require considerable manipulation skill as the cytoplast can be easily destroyed. On the other, the isolated spindle contains only a minimum of mitochondria (possibly with mutated DNA).
- Overcoming the metaphase II arrest. In some rare cases, the mature oocytes are not activated by fertilizing (injected) sperm for unknown reasons (not globozoospermia). If the cytoplasm is responsible for this aberrant behavior, then in theory, this problem can be solved by transferring the MII group into normal cytoplasts.
- Eventually, if ovulated oocytes are evidently morphologically abnormal, their metaphases II can be transferred into cytoplasts obtained by enucleation of high-quality mature oocytes.

The main advantage of MII oocyte manipulation is that oocytes at this stage are generally more available. The main problem is that they can be easily activated parthenogenetically, especially when they are aged. This can be eliminated by omitting calcium from the manipulation media. It must be also tested if MII karyoplasts can be stored in culture or in liquid nitrogen without considerable damage and used for transfer after thawing.

73.5.5 Zygotes

In fact, the transfer (exchange) of pronuclei (PNs) between mouse zygotes was the first approach demonstrating the power of micromanipulation methods [17]. From a technical point of view, it is almost similar to GV-stage oocyte manipulation. The only difference is that PN-staged embryos are very resistant to damage and PN karyoplasts fuse very efficiently to cytoplasts when electrofusion is used (when compared to GV karyoplasts × GV cytoplasts fusion). There is, however, one very serious ethical aspect when we consider the use of this approach in humans. For pronuclear transfer, we need the same developmental stage cytoplasts, i.e., one-cell-stage embryos from which their own pronuclei will be removed. This actually means the destruction of the recipient embryo (possibly this might be considered by some people as to be "a new life"). This problem can be eventually overcome by transferring both pronuclei into cytoplast prepared from parthenogenetic zygotes. It is, however, our opinion that this combination will not be widely used in human ART.

73.5.6 Cytoplasmic Transfer

The cytoplasmic transfer in human-assisted reproduction has been pioneered by Barritt and his coworkers [18]. The primary aim was to improve developmental potential of oocytes which were evidently morphologically abnormal and to enhance their developmental potential after fertilization. Thus, a certain volume of cytoplasm from healthy oocytes has been injected into abnormal oocytes either separately or along with the fertilizing sperm. This procedure led to the production of several children, some of them with mitochondrial heteroplasmy (a situation when two or more distinct mitochondrial populations coexist within one cell). This clearly demonstrates that the injected volume of healthy cytoplasm transferred cannot secure the elimination of mutated mtDNA in the patient's oocyte. Furthermore, relatively high incidence of chromosomal abnormalities and certain birth defects led to the ban of this technique in ART.

73.5.7 Transfer of Organelles

Mammalian oocytes, as nearly every cell, contain many organelles, but their separate transfer or the exchange between oocytes is very difficult because they are practically invisible. The only exception is the nucleolus (nucleolus precursor body—NPB) which is well visible, for example, in human and mouse GV-staged oocytes. At this stage, typically only one nucleolus can be observed in the GV. On the other

hand, pronuclei in human zygotes contain several nucleoli, and this, of course, complicates their manipulation. The nucleolus becomes disassembled concomitantly with GVBD, so it cannot be detected in maturing or mature oocytes, and the nucleolar material is dispersed largely in the cytoplasm. It becomes visible again after chromosomes decondense and pronuclei are formed. In humans, it has been convincingly demonstrated that the number, distribution, and position of nucleoli can serve as an indicator of further embryonic development [19]. This brings a question whether nucleoli can be eventually transferred into zygotes with abnormal nucleolar pattern, with the aim to enhance their developmental potential [20].

The nucleolus can be relatively easily removed (enucleolation) from GVs of fully grown mouse oocytes. Similarly, mononucleolar mouse pronuclei can also be enucleolated. The oocytes or embryos without nucleoli do not develop, but when the nucleolar material is reinjected into previously enucleolated oocytes (zygotes), their developmental potential is restored [21]. Interestingly, when the nucleolus is injected into an interphase cytoplasm, it is rapidly targeted into nuclei. This significantly simplifies the whole possible nucleolus transfer procedure. The nucleolus, however, is not a separated entity residing in nuclei. As we already mentioned, the close association of chromatin and nucleolus (surrounded nucleolus) is a good marker of oocyte maturation competence. This association can be also observed in zygotes. It remains to be answered if the same or equivalent association will be established after the transfer of nucleolar material and how important is this association for complete embryonic development.

73.6 Future Implications and Concerns

The aim of this chapter was not to review exhaustively the field of oocyte and embryo manipulation methods. This has been done beautifully in some recent articles and book chapters [11–13]. Thus, we rather wanted to navigate the scientists and clinicians in this field and highlight especially those articles where relevant manipulations are described in depth. Essentially, these methodological articles deal mostly with mouse oocytes and embryos, but in general, the same can be performed, of course, with some minor modifications, also in human oocytes and embryos. As we mentioned above, we strongly recommend good training with laboratory animals and ungulate oocytes. From the technical point of view, the basic micromanipulation approaches are simple, and they only need some skill. On the other hand, there are some important questions that need to be answered before these methods can be used in human-assisted reproduction. The key question is how safe are these approaches? The fragmentary information we have mostly from animal experiments is clearly insufficient. However, for example, Takeuchi et al. reported a higher incidence of abnormalities in mouse offspring originating from GV transfer oocytes [22]. It is, on the other hand, well known that the mouse is very sensitive to different manipulations and culture conditions. Similarly, the increased incidence of chromosomal abnormalities and epigenetic defects has been observed in children from oocytes injected with foreign cytoplasm. Was this the effect of cytoplasmic injection, or was this the main reason that the injected oocytes were rather abnormal? Clearly, some additional experiments with the evaluation of offspring born are necessary here. In conclusion, the recent article by Tachibana et al. [23] where metaphase II chromosomes from the cytoplasm containing mutated mtDNA were transferred into cytoplasts with normal mtDNA with two healthy offspring obtained clearly demonstrates the power of micromanipulation approaches and the area of their possible clinical application in future. The first step we can find, according to our opinion, in the paper published by Craven et al. [24] is demonstrating the feasibility of pronuclear transfer in human zygotes and describing how the reconstructed zygotes further develop in culture.

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Review Questions

1. How do the aberrations in histone acetylation/deacetylation process affect the oocyte aneuploidies?
2. What are the problems that can be solved by the nuclear and cytoplasmic transfers?
3. What are the concerns with the nuclear and cytoplasmic transfers?

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Embryo Culture and Phenotype of the Offspring

Arne Sunde

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Learning Objectives

- To know some of the data in animal models showing a relationship between the conditions of in vitro embryo culture and the phenotype of the offspring
- To know some of the data suggesting that in vitro embryo culture of human embryos may have an effect on the phenotype of the children born
- To know some of the data suggesting the composition of embryo culture media may have an effect on the phenotype of the children born
- To know other factors during in vitro embryo culture that may have an effect on the phenotype of the children born

74.1 Brief Background

Children born after assisted reproduction technology (ART) have a higher frequency of premature birth and low birth weight and a higher malformation rate, and there is evidence for an increased tendency for cardiovascular and endocrine disturbances. It is important to understand what extent is this due to maternal and paternal factors and what is the contribution of assisted reproduction as such?

In vitro fertilization (IVF) or in vitro culture (IVC) is one element of ART that is very different from what happens during natural conception. In animal models, ART has been shown to induce a shift in the phenotype of the offspring. In the human, we still need data from high-quality studies to be able to draw from conclusions.

Neither in animal nor in human studies has IVF or IVC been shown to change the genome (DNA) of the offspring. IVF or IVC does not seem to increase the frequency of mutations or chromosomal structure in the offspring. The discussion is to what extent the genetic information is used differently in the offspring. In other words, is there shift in phenotype in children born after ART? Change of phenotype here is understood as ranging from a change in the expression of genes and proteins in early embryos, changes in foetal growth trajectories, changes in physiological and endocrine parameters and psychosocial and psychomotor development. The current chapter will focus on the potential role of embryo culture and embryo culture media in modulation of the phenotype of the offspring after ART.

74.2 Influence of Nutrition of the Embryo Around the Time of Implantation

More than 50 years ago, a Norwegian county doctor working in the north of Norway made a pioneering observation. He could demonstrate that the nutritional and socioeconomic status during pregnancy in periods 1890s to 1920s had an influence on the likelihood of cardiovascular problems 40–60 years later [1, 2]. Based on this and his own observations, David Barker later formulated the DOHaD (Developmental Origins of Health and Disease) hypothesis [3]. It is now well documented that the nutritional status of the prospective mother at the time of implantations and in early pregnancy will have an effect on the health and development of the offspring [4–7]. In rodents it is demonstrated that even slight variations in the diet at the time of implantation will have an effect on cardiovascular parameters after birth [8, 9]. The composition of the intrauterine fluid is influenced by the diet of the mother changing the in vivo "embryo culture media" [10].

The (Forsdahl)-Barker hypothesis may be understood as a physiological response of the embryo to environmental factors [11]. This is most likely mediated through epigenetic change that may persist also in later generations [12–17].

In the light of the DOHaD hypothesis, one important question is to what extent will assisted reproduction including in vitro fertilization and embryo culture have an influence on the phenotype and the future health of children born.

74.3 The Effect of In Vitro Fertilization and In Vitro Embryo Culture in Animal Models

In ruminants, it is well known that fertilization and culture of embryos in vitro can lead to a change in phenotype such as increased birth weight, increased gestational length, pre- or perinatal death and large offspring syndrome [18–20]. In sheep, foetal overgrowth is thought to be due to dysregulation of the IGF2R gene [19].

In mice, in vitro fertilization results in changes in many organ systems. The effects of IVF and in vitro culture seem also to be dependent on the genetic background since different strains will show different effects. The changes observed range from change in gestational or body weight, low embryo viability, organomegaly, Type 2 diabetes and subfertility [21, 22] as well as behavioural changes such as hyperactivity [13, 14], reduced anxiety, poor spatial memory [23] and delayed preweaning and neurodevelopment [24]. In mice, a transgenerational effect on methylation of DNA and histones can be demonstrated [13, 14]. Culture of the embryos to the blastocyst stage will change the postnatal epigenome in mice including raised blood pressure and change in cardiovascular parameters.

74.4 Assisted Reproduction in Humans

Children born after IVF or ICSI have higher frequency of premature birth, lower birth weight, higher perinatal mortality and increased rate of malformations [11, 25–32]. Reduced birth weight is in general an indication of impaired foetal growth [33]. Malnutrition and smoking during pregnancy are linked to reduced birth weight and long-term effect on the health of the offspring. If the reduced birth weight of ART children per se is an indicator of long-term health consequences remains to be seen. At 2–3 years of age, the body weight of ART children is within the normal range indicating a catch-up growth in the first year of life [34].

A consistent finding in ART children is altered cardiovascular parameters. Similar to the observations in animal models, offspring after ART have shown increased blood pressure and changes in several cardiovascular parameters and suggest a cardiovascular remodelling during pregnancy that also persists after birth [35–39]. Glucose metabolism also changes accompanied with changes in insulin and IGF-I levels [40, 41]. These changes may suggest a predisposition for insulin resistance [42]. Blood lipids and body fat distribution are changed, and bone length in 7–8-year-old children is increased [35, 41, 43]. Entry into puberty seems to occur earlier in girls born after ART compared to children born after normal conception [44].

74.4.1 Change in Phenotype of the Offspring After ART in Humans: The Chicken or the Egg?

It is likely that there is a lot of factors that may contribute to the effects on the phenotype of the offspring after ART. Maternal and paternal genetic factors as well as obesity, maternal nutrition and recreational drugs may contribute to the phenotype of the offspring [45–47]. Infertility as such is associated with pregnancy complications such as pre-eclampsia, premature birth and increased malformation rate [48–52]. Ovarian stimulation and non-IVF ART will influence postnatal growth [53–55].

It is difficult to entangle the effect of ART as such from the effects of the population treated. One elegant approach to this problem is looking at couples that have two singletons, one from ART and one from a natural conception. A Norwegian registry study compared 4408 sibling singletons conceived either by ART or natural conceptions [32]. This registry contains information about confounding factors so the researchers could adjust for many of the factors that are known to influence outcomes such as gestational length, birth weight and neonatal morbidity. The study also compared the 4408 siblings with over 1.1 million children born in the same period after normal conception. The study confirmed previous observations that ART children are born earlier and have lower birth weight compared to natural conception. When comparing two singleton siblings born of the same mother, one after ART and one after natural conception, the difference was not anymore significant [32]. This study design was later used in a larger Danish study looking at 7758 siblings. They could demonstrate a small but significant difference between the ART sibling and the sibling born after normal conceptions; the ART sibling was born on average a little earlier and had a little lower birth weight [56].

ART is associated with an increased frequency of various placental pathologies such as placenta praevia [57], velamentous and marginal cord insertion [58] and pre-eclampsia and hypertensive disorder [29, 59, 60]. Using the model with sibling singletons, one after ART and the other after normal conception, Romundstad et al. could clearly demonstrate the major contributor to the increased frequency of placenta praevia is the ART procedure itself [57].

74.4.2 Is a Change in the Epigenome a Mediator of ART-Induced Changes in Phenotype?

After fertilization, large changes in methylation patterns and the epigenome take place [61], and the periconceptional environment is shown to modulate this process [62]. Embryo culture changes gene expression and phenotype [63–65]. Specifically, suboptimal culture conditions will have an influence on the epigenome of the embryo and the offspring [66], and different embryo culture media have different effects on the expression profile in embryos [67–69]. The data from animal studies are rather extensive showing that the nutrients available to the embryo around the time of implantation can have long-term consequences for development and postnatal health [8, 70]. It is likely that this is mediated through epigenetic changes, and it has been demonstrated that in vitro culture of animal embryos may have consequences for the maintenance of embryonic epigenome in the mouse [22, 71] and possibly also in humans [11, 19, 72–74]. It has been demonstrated that genes controlling growth such as IGF2/H19 and IGF2R are differently methylated after ART compared to natural conception leading to the large offspring syndrome [75–77]. Also in humans it has been demonstrated that ART led to changes in DNA and histone methylation [78, 79]. Children born after assisted reproduction show various epigenetic changes which is ascribed to the ART process [73, 80–84]. The placental epigenome is different in pregnancies after ART [85].

A study of global DNA methylation levels in the placenta of pregnancies after assisted reproduction revealed significant differences related to the conditions in the ART laboratory. Treatment-related factors like O_2 concentration during culture, conventional IVF versus ICSI, culture length (3 days versus 5 days) and cryopreserved versus fresh embryos had their specific effects on the methylation levels [86].

74.4.3 Children Born After ART: A Slightly Different Phenotype?

The changes in phenotype observed in children born after ART are not entirely similar to known syndromes or diseases such as childhood diabetes-I. Cardiovascular parameters and glucose metabolism suggest an increased long-term health risk, while the blood lipid profile an body fat distributions suggest the opposite. It is therefore difficult to predict the health profile of ART children when they become 50–60 years old. They may be similar to children exposed to famine in early foetal life; they may have a health profile within the normal range or have a health profile different from what we have seen until now, but not necessarily to the worse [41, 87].

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74.5 In Vitro Culture of Human Embryos, an Effect on Phenotype of the Offspring?

Available evidence from animal and human studies shows that in vitro fertilization and embryo culture are associated with phenotypic changes. Putative contributors to this are procedures such as ICSI and cryopreservation, physiochemical factors, pollutants and the composition of embryo culture media (Table 74.1).

74.5.1 Embryo Culture

74.5.1.1 Gas Phase

In the fallopian tube, the oxygen concentration is around 5% and lower that in the ambient air. In the early days of IVF, fertilization and embryo culture was performed with reduced oxygen concentration. This was abandoned, largely out of convenience and cost and the majority of clinics culture embryos with 20% oxygen. Currently, there is accumulating evidence that the culture in 5% oxygen gives better development rates, better embryo quality and, in some studies, better pregnancy rates compared to 20% oxygen [88–94]. Similarly to other cell types, embryos have a system that regulates expression of many genes in response to the oxygen concentration in the cell [95–98]. In human embryos, the gene expression profile in embryos cultured at 20% O_2 is different from that of embryos cultured in 5% O_2 [69]. A complicating factor is that it seems that the effect of varying the O_2 concentration on the gene expression profile of human embryos also is dependent on the culture media used [68].

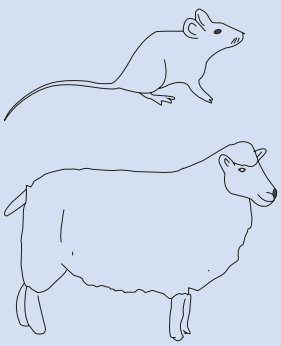
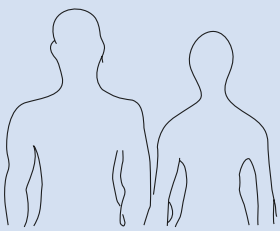
74.5.1.2 Temperature

In human IVF, gametes and embryos are exposed to changes in temperatures and pH that they do not encounter in vivo. Oocytes are very sensitive to reduced temperatures due to instability of the meiotic spindle. Already at temperatures around 30 °C, the spindle breaks down and shows limited capacity to reassemble following warming up to 37 °C again [99, 100]. Zygotes and embryos tolerate temperature fluctuations better, but each time the temperature is outside the physiological range, embryo metabolism is affected. Data suggest that in vivo, the temperature in the female reproductive tract is slightly lower than 37 °C [101]. A prospective randomized study comparing culture at 36 or 37 °C did give different results in terms of blastocyst development rates [102].

74.5.1.3 pH

The pH in the fallopian tube changes throughout the menstrual cycle, 7.1–7.3 in the follicular phase and rising in the luteal phase due to accumulation of HCO_3^+. It may be argued that early embryos are adapted to a high rather than a low pH. The internal pH in oocytes is 7.4, and cleavage-stage embryos have less capacity to compensate for acidic

Table 74.1 Summary of effects of in vitro fertilization and in vitro embryo culture on the phenotype of the offspring in two animal species and humans

Species	Intervention	Effect
Rodents and ruminants 	In vitro fertilization and in vitro culture	Effects on epigenome including transgenerational effects Changes in foetal growth, gestational length and birth weight Low embryo viability, increased perinatal morbidity and mortality Organomegaly, Type 2 diabetes, changes in cardiovascular parameters, behavioural changes
	Composition of culture media	Influence on embryo epigenome
Humans 	In vitro fertilization and in vitro culture	Reduced gestational length and birth weight Increase in placental dysfunction
	Extended in vitro culture (blastocyst)	Increase in birth weight, increased malformation rate
	Cryopreservation of embryos	Influence on embryo epigenome Increased birth weight, increased perinatal morbidity
	Culture with 20% O_2 compared to 5% O_2	Influence on embryo epigenome
	Composition of culture media	Influence on embryo epigenome Influence on birth weight

challenge than alkaline challenge. It is only at the blastocyst stage that embryos attain the capacity also to compensate for acidic challenges [103].

It is still unclear what effect, if any, the transient changes in temperature and pH have on the phenotype of embryos or the offspring. One can argue that environmental stress in general is less desirable and should be avoided.

74.5.1.4 Air Quality and Organic Pollutants

In animal models, a variety of organic pollutants have been shown to induce a shift in the phenotype of the offspring. A classical model is a mouse where the expression of the Agouti gene is regulated by methylation in the promotor region. This model can be used to look for factors that may change imprinting and gene expression during embryo culture [65, 104]. Bisphenol A is an endocrine disruptor and modulator of gene expression and will influence the phenotype of the offspring if present at sufficient concentrations in the culture period [104]. Bisphenol A is almost ubiquitous, and the concentration in the urine of women undergoing IVF has been related to their reproductive health [105]. Bisphenol A is used in the manufacture of a variety of plastic components including polycarbonate plastic intended for in vitro culture. To what extent this has been or is an issue for IVF laboratories is not known, but at least when using culture ware intended for human IVF, bisphenol A concentration during embryo culture seems to be low [106]. Volatile organic compounds (VOCs) are present in ambient air. Urban air and indoor air may contain concentrations of these components high enough to influence growth of embryos in vitro. Each IVF lab must evaluate the quality of the air in the laboratory as well as any gas used for the incubators [107] .

Culture Length

Days in culture may have an effect on phenotype. Several reports indicate that there is a difference between transfer of cleavage-stage embryos and blastocysts. Concerning implantation rates, many clinics claim that they have higher implantation rates after blastocyst transfer compared to cleavage-stage transfer. This may increase the rate of elective single embryo transfer that has been clearly shown to be beneficial for the health of the offspring [108, 109].

An argument against blastocyst transfer is the slightly higher incidence of pregnancy complications and concerns for the health of the offspring. In pregnancies following blastocyst transfer, the frequency of pre-eclampsia is moderately increased compared to pregnancies after transfer of cleavage-stage embryos [110]. There is an increased frequency of preterm birth [111, 112], but despite this, the birth weights are slightly higher, and the frequency of offspring large for gestational age (LGA) is increased following blastocyst transfer [113, 114]. This difference may be partly explained by the skewed sex ratio in favour of males [115, 116]. The rate of congenital malformations is slightly higher after blastocyst transfer [117]. Published data are not in agreement concerning a putative increase in the rate of monozygotic twins after blastocyst transfer [118]. It is possible that this conflicting result is that the rate of monozygotic twins is dependent on culture factors [119]. On the positive side, blastocyst culture allows embryos to be self-selected. The ability to grow to a normal blastocyst with good morphology is a sign of embryo competence. If it is indicated to perform embryo biopsy, a trophectoderm biopsy at the blastocyst stage is less harmful to the embryo than removal of a blastomere at the cleavage stages [120]. High survival and post-warming implantation rates have been obtained after vitrification of blastocysts [121]. For many ART clinics, the positive sides of blastocyst culture are perceived to be more important than the negative aspects, and culture to the blastocyst stage is the preferred option.

Cryopreservation

Gene expression studies have found a difference between cryopreserved/thawed embryos and fresh embryos [122]. A consistent finding is that transfer of cryopreserved embryos (FET) is associated with an increase in birth weight, an increase in the frequency of macrosomia and offspring being large for gestational age (LGA) [113, 123, 124]. Despite the fact that the body weight of children after FET is more similar to children born after normal conception, they have an increased frequency of perinatal morbidity and mortality compared to children after normal conception [49, 124, 125]. A study of singleton siblings born after ART, one after transfer of fresh embryos and one after cryopreserved embryos, shows clearly that it is the cryopreservation of embryos that is associated with the increased birth weight of the offspring [126].

Initial reports indicated that pregnancy and delivery complications were more frequent after transfer of vitrified blastocysts compared to slow-freeze [127]. Later reports could not confirm this, and vitrification of blastocyst does not seem to negatively affect pregnancy and delivery complications and neonatal health [128–130].

74.5.2 Embryo Culture Media

The embryos undergo a complete remodelling of its genome and epigenome during development from fertilization to the blastocyst stage. At the blastocyst stage, cells are allocated to the trophectoderm (TE) and the inner cell mass (ICM) lineages [83]. Culture condition including culture media may modulate these events and have an influence on the foetal growth and subsequently the birth weight.

All cells, including early embryos, have the ability to adapt their gene expression and metabolism to the available nutrients. Amino acids and glucose are particularly important, using the AMP-activated and mTOR-related kinases to exert the effect on the epigenome [131, 132]. A culture media low in nutrients may induce epigenetic changes that promote a thrifty phenotype. When the embryo is transferred to the high-nutrient environment in the uterus, this may lead to overgrowth. The reverse may also occur when the in vitro culture media are very rich in nutrients.

In the beginning, culture media for human IVF ranged from simple salt solutions such as Earle's or Tyrode's solution, quite often prepared by the ART laboratory, to commercially generic tissue culture media such as HamF10. These media were usually supplemented with serum or protein extracts of animal or human origin. It is perhaps surprising that even after nearly three decades of commercial production of embryo culture media, there is little standardization. Many ART laboratories use combinations of culture media from different manufacturers: aspiration media from one manufacturer, fertilization media from a different manufacturer and cleavage stage from still another source, etc. This is unfortunate since most manufacturers try to harmonize their different products to avoid a too abrupt change in the composition of the environment when moving zygotes and embryos from one step in the process to the next. Mixing of different media systems also makes it difficult to evaluate a relationship between the composition of a given culture media/system and phenotype of the offspring.

Commercially available culture media vary widely in their composition [133]. ◘ Table 74.2 shows the composition of two commercially available embryo culture media; one of them contains 13 ingredients and the other over 80 ingredients. These ingredients are generally added as various salts, and the concentration of each of them might vary between the commercially available culture media. These culture media differ in the content of substances like amino acids and vitamins which is known to influence methylation levels and the epigenome [134, 135]. With such a big difference in the composition of culture media, it should come as no surprise that the embryos respond differently when it comes to expression of genes. This is clearly illustrated in a study by Kleijkers et al. Human zygotes and embryos were prospectively randomized to be cultured in either human tubal fluid (HTF), a relatively simple culture medium, or Vitrolife G5, a modern relatively complex culture medium. Embryos not selected for transfer or cryopreservation at Day2/3 were cultured to Day 6 and then analysed for genome-wide gene expression [68]. ◘ Figure 74.1 shows the differential gene expression in embryo culture in the two culture media in the study of Kleijkers et al. Only genes with a differential gene expression above 5 (5 times more) or below 5 (5 times less) in embryos cultured in G5 compared to embryos cultured in HTF are shown in ◘ Fig. 74.1. It is still unknown whether such a difference in the gene expression profile blastocyst cultured in vitro has any long-term consequences for the health of the offspring.

From animal experiments, ammonium build-up in culture media may have an effect on the phenotype of the offspring [136]. The ammonium stems from breakdown of amino acids, glutamine in particular. This is primarily a non-enzymatic reaction that occurs faster at 37 °C than at 4 °C. One way of alleviating this problem is to add glutamine as a dipeptide which is chemically more stable [137]. Looking at modern embryo culture media, the rate of ammonium build-up at 37 °C varies considerably between different media from negligible to a rapid increase of ammonium up to levels known to have a potential influence on the offspring [138]. This fact should be taken into consideration concerning choice of both culture media and of culture protocols.

◘ **Table 74.2** The chemical composition of two commercially available embryo culture media. One "simple" in the sense that it contains few ingredients, the other "complex" with many ingredients. Protein supplements such as hSA are added to both culture media

Simple	Complex						
Bicarbonate	Adenine	Cobalt	Folic acid	L-Aspartate	L-Threonine	Pantothenate	Sulphate
Calcium	Ascorbic acid	Copper	Gentamicin	L-Cysteine	L-Tryptophan	Penicillin	Taurine
Chloride	Aurintricarboxylic acid	Cyanocobalamin	Guanine	L-Glutamic acid	L-Tyrosine	Phenol red	Thymine
Citrate	Acetic acid	Citrulline	HEPES	L-Glutamine	L-Valine	Phosphorus	Thiamin
D-Glucose	Aluminium	Chromium	h-r-Insulin	L-Glycine	L-Ala-L-Glut	Potassium	Thioctic acid
Magnesium	Bicarbonate	Cytosine	Hypotaurine	L-Histidine	Linoleic acid	Putrescine	Uracil
Penicillin	Calcium	D-Biotin	Hypoxanthine	L-Isoleucine	Magnesium	Pyruvate	Vanadium
Phenol red	Citrulline	D-Glucose	Inositol	L-Leucine	Manganese	Pluronic-F-68	Zinc
Phosphate	Cholesterol	D-Pantothenate	Iron	L-Lysine	Molybdenum	PVP 10	
Potassium	Choline	EDTA	Lactate	L-Methionine	Nickel	Pyridoxine	
Sodium	Chloride	Ethanol	L-Alanine	L-Phenylalanine	Nicotinamide	Riboflavin	
Streptomycin	Citrate	Ethanolamine	L-Arginine	L-Proline	Ornithine	Selenium	
Sulphate	Cobalamin	Estradiol	L-Asparagine	L-Serine	Maleic acid	Sodium	

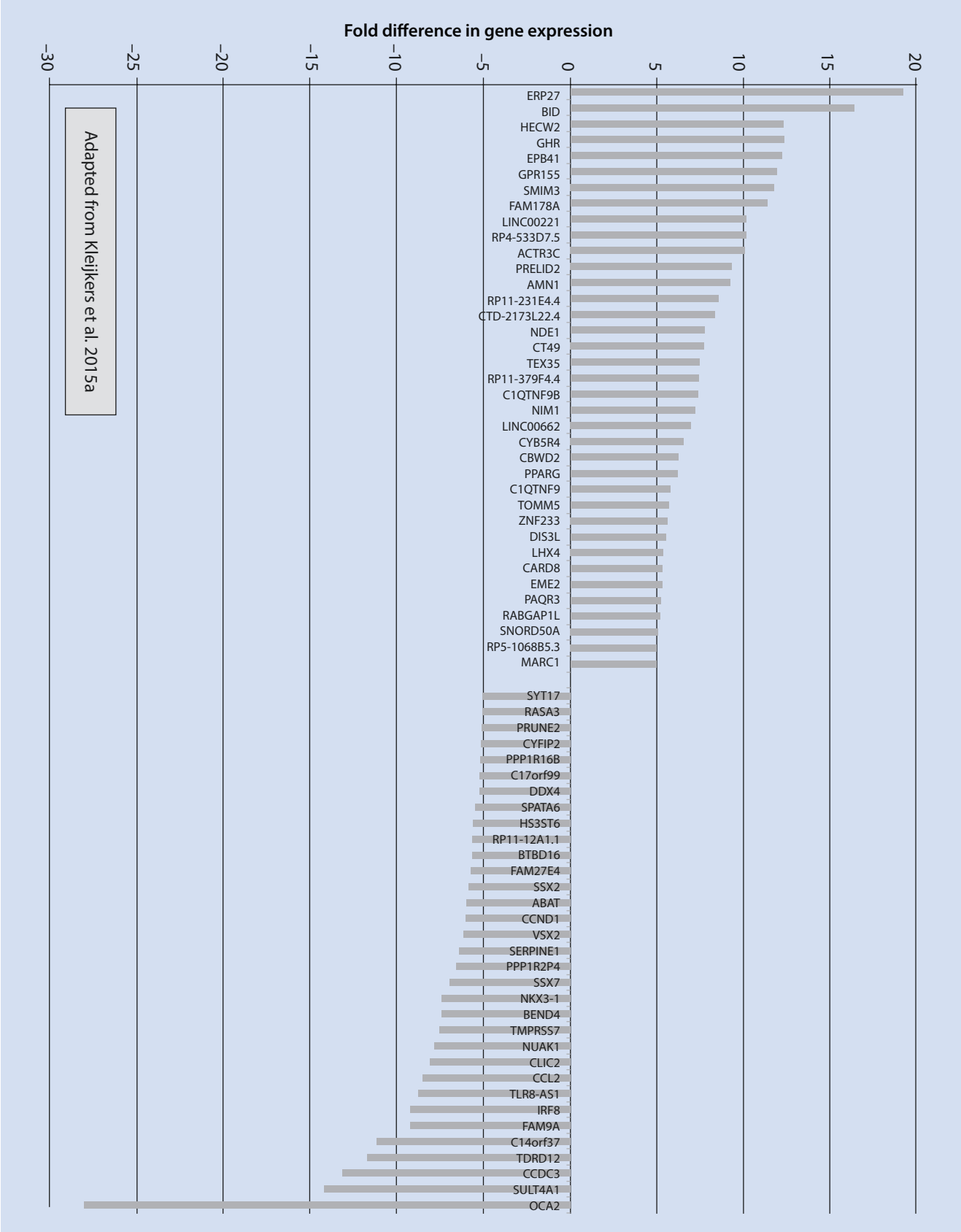


Fig. 74.1 Differential gene expression between blastocysts cultured either in Vitrolife G5 Plus or human tubal fluid (HTF). Only genes showing a more than fivefold difference in gene expression are shown in the figure. Positive values: five higher expressions in embryos cultured in G5 relative to embryos cultured in HTF. Negative values: expression lower in embryos cultured in G5 compared to HTF. (Adapted from Kleijkers et al. [68])

In some commercially available culture media, peptide growth factors such as insulin and granulocyte colony-stimulating factor (GC-CSF) have been added in relatively high concentrations. Growth factors may alter embryonic growth and lineage allocation to the inner cell mass (ICM) or trophectoderm (TE). In animal models, the presence of insulin in the embryo culture media will change the expression of imprinted genes [139]. In vivo, embryos are exposed to many growth factors in a timely fashion both concerning concentrations and timing in relationship to developmental status. Deliberately adding potent growth factors in superphysiological concentrations to embryo culture media should therefore be done with utmost care.

Most embryo culture media are supplemented with protein supplements. These supplements also vary in their chemical composition and will contain variable amounts of bioactive molecules [140, 141]. Commercially available protein supplements usually contain octanoic acid as a protein stabilizer. In animal experiments, octanoic acid is demonstrated to influence the phenotype of the offspring when used in the same concentrations as in human embryo culture [142]. Moreover, the source of protein supplements may also influence the phenotype of the offspring. In a retrospective study, Zhu et al. noticed that the birth weight of the offspring was related to the protein source added to the embryo culture media [143]. A protein supplement such as hSA contains amino acids including glutamine. Ammonium build-up is one possible explanation for the observation that the birth weight of the offspring seems to be related to the time since the culture medium was produced. Birth weight of children from embryos that have been cultured in medium close to the expiry date was lower than when embryos were cultured in freshly produced media [144].

74.5.2.1 Embryo Culture Media and the Phenotype of the Children Born

Most retrospective studies have found no differences in birth weights or other characteristics of the offspring related to the culture used for IVF/ICSI. The design and the control groups vary between these studies, and it is difficult to evaluate to what extent one could control for known confounders in these studies. See extensive review by Zandstra et al. [145].

A Danish retrospective study comparing the outcome after IVF/ICSI with different embryo culture media could not find any differences in birth weights. They however reported a significant relationship between birth length and the culture media used [146]. One would assume that Ponderal Index (measure of leanness) also was related to the culture media. In a large registry study, Eskild et al. could demonstrate the different culture media had different effects on placental weights, birth weights as well as placental/birth weight ratio [147].

It is evident that there are many confounding factors when one tries to correlate the chemical composition of a given culture media and the phenotype of the offspring. Many of the known confounders have been mentioned earlier in this chapter. Additionally, it is reasonable to assume that there are confounders yet to be identified. The same challenges face almost any study of a given intervention in ART and the development and health of the offspring. A way around this problem is to do properly designed controlled prospective randomized studies, the gold standard being a multicentre prospective randomized double-blind study, a complicated, laborious and expensive study design but widely recognized as the best concerning controlling for confounders in clinical trials.

One prospective randomized study comparing two single-step media (SSM; Irvine and Global, Life Global) was stopped prematurely due to inferior implantation rates in the SSM group. The children born as a result of the study were followed up until the age of 4.

A worrying find in this study is that psychomotor development of the children differs according to the embryo culture media used [148].

A few properly designed prospective randomized studies looking at a possible effect of embryo culture media on the birth weight of the offspring have been published (Table 74.3). The first study that demonstrated a difference in birth weight was that of Dumoulin et al. published in 2010 [149]. In this study they detected a 245 g difference in birth weight and a significant difference in Z-score between children born after IVF/ICSI and embryo culture either in Vitrolife G1.3 or Cook K-SICM [149]. Carrasco et al. later did a similar, but smaller, prospective study looking at the same two culture media [150]. In this study, the differences in birth weights and Z-score were in the same direction but smaller than that found in the study by Dumoulin et al. and failed to reach significance. Ziebe et al. did a large multicentre study comparing Medicult Embryo Assist with or without added GM-CSF and could not see any differences in birth weights [151]. In 2016, Kleijkers et al. published the outcome after a large multicentre prospective randomized double-blind study conducted in the Netherlands [152]. In this study, a total of 836 couples undergoing IVF/ICSI was prospectively randomized to have fertilization and embryo culture carried out in two different culture media: Vitrolife G5 Plus or human tubal fluid (HTF) supplemented with 10% albumin. Comparing the birth weights of the singletons born, there was a significant difference of 181 gram and a significant difference in the Z-score. Children born after the embryos have been cultured in HTF were the heaviest [152].

Not all studies looking at a possible contribution of the embryo culture media used to the birth weight of the children found effects. This is not surprising. Some of the retrospective studies performed had suboptimal design, and some of the prospective studies were powered only to detect fairly

Table 74.3 Prospective randomized studies looking at possible effect of embryo culture media on the birth weight of children after IVF

Study	Design	Media comparisons	Number of children included	Outcome	*P* value
Dumoulin et al. (2010)	Single centre, open, prospective randomized	Vitrolife G.1 Cook K-SICM	110 78	Birth weight different	$P < 0.005$
Carrasco et al. (2013)	Single centre, open, prospective randomized	Vitrolife G.1 Cook K-SICM	49 49	No difference in birth weight	Not significant
Ziebe et al. (2013)	Multicentre, double blind, prospective randomized	Medicult Embryo Assist with GM-CSF added Medicult Embryo Assist no GC-CSF added	163 141	No difference in birth weight	Not significant
Nelissen et al. (2012)	Single centre, open, prospective randomized	Vitrolife G.1 Cook K-SICM	168 126	Birth weight different	$P = 0.006$
Hassani et al. (2013)	Single centre, open, prospective randomized	Medicult ISM1 Vitrolife G1.5	86 78	Birth weight different	$P < 0.001$
Kleijkers et al. (2016)	Multi centre, double blind, prospective randomized	Vitrolife G5 HTF	184 159	Birth weight different	$P = 0.005$

large differences. It is also entirely conceivable that embryo culture media from two different manufacturers are quite similar in their composition and are least when it comes to ingredients that may have an influence on birth weight. Comparing these two culture media would not reveal any difference. Additionally, birth weight might not be the best estimate to show a culture media-related effect on the phenotype of the offspring [146].

74.6 Summary

In summary, available evidence suggest that embryo culture media may have an effect on the epigenome of embryos, the growth trajectory of the foetus and the birth weight of the child (Fig. 74.1, Table 74.3). It is however far too early to predict the long-term effect on health of the children born. One of the unknowns is the relevance of the shift in birth weight induced by certain embryo culture media compared to other well-known factors like cigarette smoking and malnutrition that also have an influence on foetal growth. Furthermore, we do not have information about which of the ingredients in an embryo culture media that may modulate birth weight or whether the effect(s) are related to the concentration of some key ingredients. Most manufacturers of embryo culture media do not give full information about the composition of their media. This makes it almost impossible to formulate hypothesis concerning cause and effect concerning the relationship between embryo culture and the phenotype of the offspring. This is one of the reasons why the European Society for Human Reproduction and Embryology advocates full transparency concerning the composition of embryo culture media [153].

Review questions

1. What is the likely mediator of the effect of environmental factors around the time of implantation?
2. What is the basic rationale behind the DOHaD hypothesis?
3. Does the composition of embryo culture media play any role in early embryo gene expression?
4. Is the composition of commercially available culture media harmonized?
5. What is the relevance of the DOHaD hypothesis for evaluating the composition of embryo culture media?
6. What is the effect of cryopreservation of human embryos on the birth weight of children born?

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Traceability in ART

Tom Beckitt and Tammie Roy

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Learning Objectives

- To introduce the reasons for traceability in assisted reproductive technology (ART) laboratories and the primary reasons for its importance
- To discuss the general concepts of manual and electronic traceability systems
- To provide an overview of the features included within some of the currently available electronic systems
- To present the general expectations of electronic traceability systems
- To propose potential future developments of, and additions to, electronic traceability systems and the value that each progression will bring to the field of ART

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75.1 Reasons for Traceability in ART

Traceability procedures, including witnessing of processes and sign-off by additional staff members, are integral practice within ART laboratories worldwide.

Traceability is important for the reassurance of laboratory and clinical staff and for maintaining efficient workflow, but in a heavily regulated sector, it is also required to conform to regulations set by national and international authorities. Auditing by regulatory bodies is commonplace, and any instances which signify a failure to comply can result in considerable penalties and, in cases where major non-conformances are discovered, in the closure of ART clinics.

Without the use of adequate witnessing procedures, there have been instances of severe mistakes which have led to the implantation of the wrong embryo and the development of a foetus inside the incorrect patient.

While these major non-conformances are rare and almost all ART clinics consistently adhere to regulations, ensuring compliance using manual, non-electronic systems is often cumbersome and time-consuming. Furthermore, manual witnessing is not the most efficient method when considering laboratory workflow because it does not allow the additional possibility of monitoring activities in the laboratory as part of the standard witnessing procedure.

In contrast, electronic methods not only assist laboratories with adhering to regulations but also provide online, workflow monitoring, enabling staff to see the impending tasks and monitor their progress, providing managers with a tool to assist with scheduling those tasks. In recent times, there have been noteworthy additions to the functionalities included in electronic systems, which have further improved laboratory processes.

75.2 Methods of Traceability in ART

In ART laboratories worldwide, traceability is maintained through the use of either manual procedures or electronic systems.

75.2.1 Manual Procedures

Most manual procedures of witnessing in ART laboratories involve multiple identification of samples by two or more trained staff. Every time sperm, oocytes or embryos are moved, additional employees are required to be able to confirm that each handling step is performed correctly and that the dishes, tubes and other consumables are correctly selected.

Not only are methods such as this time-consuming (often requiring additional signage and paperwork compared with electronic systems), and somewhat inefficient, they also create additional risks in laboratories, such as interrupting other scientists in the course of their duties to identify a sample, or lead to difficulties when minimal staff are working during quiet periods. In the latter of those situations, the presence of a manual witnessing system can mean that additional staff are present for the sole reason of witnessing procedures. This can be costly and, for small clinics, can be a regular occurrence, leading staff to seek less quality-focused methods of overcoming the requirement for witnessing. An example of this is where some laboratory staff check labels on consumables once, look away for a few seconds and then look again to confirm that they are correct. Evidently, this type of process increases the likelihood of human error and of 'involuntary automaticity', where errors occur because laboratory staff see what they expected to see [1]. Therefore, manual procedures should be avoided wherever possible by the implementation of electronic witnessing systems.

75.2.2 Electronic Systems

Electronic witnessing systems aim to further reduce the risk of error during sample handling (increasing peace of mind for laboratory staff) and have the secondary purpose of enabling laboratory workflow to be electronically monitored, ensuring maximum efficiency. There are two types of electronic witnessing, which include the use of either Radio-frequency Identification Labels (RFID) or barcodes and compatible scanners.

75.3 Currently Available Electronic Systems

There are several options available on the market to satisfy the needs of customers wishing to implement an electronic witnessing system, utilising both RFID and barcodes, some of which are briefly summarised below:

RI Witness™ is a product available from Research Instruments which automatically tracks and records data across the whole laboratory using RFID tags contained within consumables and readers contained within microscope stages and incubators. At each work area, a small LCD touchscreen is available to monitor workflow and ensure that traceability of sperm, oocytes and embryos is maintained. Furthermore, media product containers are also allocated an RFID tag,

which ensures that the correct media is used for each and every stage of ART cycles. RFID is unique to RI Witness, and although this innovation has its advantages, it also requires a significant investment from the clinic, both financially – to fit RFID readers into existing equipment – and in terms of downtime, while equipment is adapted and laboratories alter their workflows to incorporate the new system. Clinics must also purchase specific consumables or self-adhesive RFID tags, and as those RFID tags cannot be stored in liquid nitrogen, a separate system must be employed to monitor traceability of cryopreserved samples [2].

Matcher, developed by IMT International, is a barcode system which integrates the use of digital photography within the laminar flow hoods where procedures are performed to capture all of the activities that occur during each ART procedure. At each stop point of the cycle, the consumables are scanned to ensure traceability and ensuring that the correct dishes, tubes and media are being used. This system also has options for workflow management and offers flexibility, as the barcodes can be attached to any existing lab consumable [3].

Trusty™, by Optimal IVF, consists of software which can be loaded onto computers and used to assign a unique number to any patient cycle. This unique ID is then translated to a barcode, which can be printed onto multiple labels for use at all points of ART cycles. Barcode scanners are purchased separately and used to ensure each consumable is correctly selected. The system also eliminates the requirement for paper-based systems by collating all information onto the stand-alone system to maintain traceability [4].

The OCTAX Ferti Proof system by Vitrolife monitors and logs all actions during an ART cycle using a QR code scanning system. As scanning progresses throughout the day, batch numbers of consumables are recorded, and reports can be generated accordingly, in order to monitor workflow. Similarly to the other electronic systems available on the market, Ferti Proof removes the need for paper-based documentation. This system requires some printers and scanners to be purchased and distributed throughout the clinic, and thus there is some capital investment required; however, the overall amount required is significantly less than for an RFID-based system [5].

Gidget, available from Genea Biomedx, is the most recent addition to the market. The system utilises both QR codes (2D) for laboratory consumables and linear barcodes for cryodevices, and hence, labels can be scanned after cryostorage. The system is also completely portable and can be used anywhere in the laboratory, or indeed across other departments, with handheld scanners being carried by laboratory staff and linking via Wi-Fi to a server. The details which have automatically synchronised to the server can then be displayed on screens in the laboratory to show, in real time, the status of each patient currently on cycle, adding to the visibility for staff and managers and allowing them to further monitor and enhance workflow. Full audit reports can be generated from the synchronised data, within which each session in the system is allocated a unique ID number. In addition, the Gidget system is fully customisable so that individual laboratories can incorporate their standard procedures and workflow into the system, meaning that the system is personal to the processes in each laboratory, and further, it is adaptable to laboratories, so it does not require additional equipment to be purchased or contain fixed rules which cannot be modified by users or Laboratory Managers. This adaptability extends to the possibility for staff to use any desktop or laptop computer which is registered on the server to add or modify patient entries, as well as on the handheld devices. The adaptability aspect of the system also means that there is a relatively low cost involved with its implementation.

Although the system relies on the use of QR codes and barcodes, there is also the presence of a manual witnessing functionality if the labels containing these are damaged in any way, where identification by two staff can be performed. Although this is similar to a manual procedure, both of the staff members involved in the double identification must scan their personal QR code so that the system records that the process was performed correctly and automatically include it within the audit report.

In relation to label mismatches, the possibility exists for user accounts to be logged out and the patient's cycle locked if they mismatch two patient identification labels, at which time their supervisor or other specified management personnel with a higher access level to the Gidget system would need to reinstate their access and, if desired, add comments to explain the reason for 'unlocking' the cycle that will be visible in the audit report. This is beneficial because it prevents users from manually overriding their mistake, or indeed from the failure actually occurring, and can also highlight if specific personnel require additional training. For minor breaches which do not constitute a failure in the process or any risk to the patient, such as forgetting to scan the lid of an embryo-containing dish, a warning is displayed on the handheld device. This means that laboratory staff are made aware of errors and can ensure to correct them, without the general workflow being unnecessarily adversely affected to a significant degree.

A disadvantage of the system is that it is not possible to upgrade 'over the air' to new versions once the system is purchased, meaning that a skilled service technician must visit to upgrade software, contributing to an additional cost for the clinic. It is likely that in future releases of the system, over-the-air upgrades will be possible, allowing clinics to upgrade to the latest software at their convenience and with minimal associated cost.

75.4 Expectations of an Electronic Traceability System

The introduction of electronic traceability systems into laboratories should be seamless, further supporting the requirement for adaptable systems which are ready for 'go-live' as soon as they are switched on to minimise downtime and which do not entail purchasing of additional or replacement

equipment. They should save time over manual methods which they are intended to replace, make audits easier to perform and enable trained scientists to work in a smarter, more streamlined manner.

Ultimately, the use of the system should provide a secondary, automatic pair of eyes, hence reducing the potential for human error and ensuring compliance with regulatory guidelines and legislation. It should also, at a very minimum, capture 'near misses' in laboratory processes to identify the more error-prone elements of workflows, enabling laboratory managers and directors to consistently improve the quality of their systems and procedures. The overall benefits of a system which provides a solution such as this are for laboratory staff to have complete peace of mind, for Laboratory Managers and Directors to consistently improve efficiency in their workflows and for both groups to ensure that traceability is of a standard which leads to audits being passed every time.

75.5 Future Developments in Electronic Traceability Systems

Further to the current expectations of an electronic traceability system for ART laboratories, there are also several, exciting prospects which could be added in the future.

These possibilities are far and wide but in particular, laboratory workflows could be streamlined more effectively and processes made more efficient with the addition of inventory tracking, where consumables such as media and oil would be scanned when used and customisable warnings given to users when stock was becoming low or when batches were about to expire. The same data obtained for this purpose could be utilised elsewhere, such as for monitoring oil and media consumption in order to reduce cost or to highlight if clinical endpoints have been detrimentally affected since the introduction of a specific batch of consumable. Tasks performed by scientists could also be monitored by Laboratory Managers to determine where additional training may be required, therefore increasing overall efficiency.

A secondary addition to any of the aforementioned systems could be that of cryostorage monitoring. A feature which is to be included in the next version of Gidget, Genea Biomedx' electronic traceability system, is the monitoring of embryos which are stored in liquid nitrogen. The handheld devices will not only be able to monitor inventory but will also monitor the available capacity of liquid nitrogen tanks and the temperature of each specific embryo-containing cryodevice. Moreover, the specially designed handheld device which is used to pick up containers from within the liquid nitrogen will flash green when the correct vessel has been selected and flash red when it has not, therefore reducing human error and eliminating the possibility of collecting the wrong embryo for thawing.

As we move forward with the use of pre-implantation genetic diagnosis, the possibility of tagging individual embryos and/or tissue is perhaps an exciting prospective option. At present, traceability systems are often patient-specific rather than embryo-specific, thus making it difficult to monitor the fate of embryos throughout the whole confluent of additional or complementary procedures.

Overall, the workflow aspect of electronic traceability systems can be improved further, with the inclusion of options for displaying preparatory tasks and giving Laboratory Managers the ability to allocate resources at the correct times and to the correct tasks. With some companies' options, there is the possibility of linking the electronic traceability system with other equipment. In the case of Genea Biomedx, this includes the Geri Connect and Geri Assess products, the former of which allows remote monitoring of embryos contained within Geri incubators and the latter which allows both manual and automatic annotation of embryos based on morphokinetic parameters, therefore enabling the screen in the laboratory which highlights workflow to also display details of embryos' fate and the resultant impact on workflow. Additionally, laboratory workflows can be considerably enhanced by the integration of traceability systems with electronic medical record (EMR) systems that clinics already have in place, ultimately leading to a more streamlined, efficient workflow, allowing for greater control of quality procedures and significant time and cost savings.

Review Questions

1. Why is traceability required, and why is adequate control of witnessing procedures important?
2. What options are available for ensuring traceability in ART laboratories?
3. Which electronic systems exist for ensuring traceability is conducted properly and efficiently?
4. What should a clinic expect from their chosen electronic traceability system?
5. How could the current electronic traceability systems be expanded, and what possibilities exist for future development?

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Molecular Reproduction

Contents

The Role of Mitochondria in the Establishment of Developmental Competence in Early Human Development

Jonathan Van Blerkom

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Learning Objectives

- To recognize the pleiotropic roles mitochondria play in the maintenance of normal cell function
- To recognize which roles operate during human oogenesis and preimplantation embryogenesis to establish or compromise developmental competence
- To understand the meaning and role(s) of cellular energy expressed primarily in the form of mitochondrial-derived ATP as a primary driver of developmental competence for the human oocyte and preimplantation embryo
- To understand the difference between mitochondrial mass and mitochondrial DNA content as they relate to competence

While morphological and performance characteristics of oocytes and early human embryos during culture in vitro have long been the mainstay of clinical IVF for evaluations of developmental competence, new technologies based on genomic, proteomic, and metabolomic analysis have begun to enter assessment schemes by providing a wide array of target genes whose expression levels, measured at the mRNA and protein level with commercial and custom-made microarrays, may be competence associated. The relative merits of morphology and different "omic"-related methods for competence assessment and selection remain to be determined from comparative outcome findings. Whether they can be readily incorporated into the IVF laboratory and whether quantitative measurements at the biochemical and molecular levels can provide a level of confidence that is superior to what an experienced observer would normally conclude from detailed microscopic findings. However, morphology and metabolomics are the most likely to be developmentally related and to reflect the normality of molecular and cellular functions during oogenesis and preimplantation embryogenesis.

An understanding of the biological origins of developmental competence in the oocyte and how it is maintained through the pre- and early postimplantation stages of human embryogenesis has been an ongoing theme of research in clinical IVF. More recently, the notion that mitochondria may be involved in human oocyte and preimplantation embryo competence has led to a resurgence of interest in the role of this cytoplasmic component as a central player in the regulation and normality of early human development. While the respiratory role of mitochondria as the site of oxidative phosphorylation and ATP production is universally recognized by the common use in the literature of terms such as "the powerhouse of cells," these organelles have pleiotropic functions including β-oxidation, steroidogenesis, reactive oxygen species (ROS) generation, oxygen sensing, and participation in the regulation of calcium homeostasis, signal transduction, determination of cytoplasmic redox state, and apoptosis [1–3]. For the oocyte and embryo, specific mitochondrial parameters such as mtDNA copy number and cytoplasmic bioenergetic levels have been correlated with or related to the frequency of aneuploidy and the success of fertilization and preimplantation embryogenesis [4–12]. Given the multiple and varied roles that mitochondria have in normal cell function, it is not too surprising that they are an ideal candidate as a developmental common denominator, for competence studies in human oogenesis and embryogenesis and as such form the basis for this review.

76.1 Mitochondrial Functions and Activities in the Mammalian Oocyte and Early Embryo

Mitochondria in the mammalian oocyte and early preimplantation-stage embryo, including the human, are functionally similar but structurally undeveloped when compared to forms present during the blastocyst stage or in somatic cells, such as those of the cumulus oophorus and corona radiata. Human oocyte mitochondria are usually spherical to slightly oval in shape and about 1 μm or less in diameter [13, 14]. While active in ATP synthesis, the levels of generation are assumed to be relatively low, which would be consistent with the occurrence of few short cristae located at the periphery of an electron-dense matrix. Prior to cavitation, mitochondria usually become elongated and progressively develop well-formed cristae that traverse a matrix of lower electron density. By the expanding blastocyst stage, they assume forms characteristic of highly active (energetic) organelles typical of differentiated cells. An early indication that the normality of mitochondrial development may be a factor in the inability of cleavage human stage embryos to initiate cavitation comes from fine structural studies of human embryos that arrest development during cleavage, in which few, if any, stage-appropriate forms are detected [15, 16]. Although electron microscopic findings provide an intriguing correlation with developmental arrest in the human, a cause–effect relationship cannot be concluded solely on the basis of fine structure.

76.2 Developmental Significance of Mitochondrial DNA Content

More recent investigations of the involvement of mitochondria in human oocyte and embryo competence have correlated stage-appropriate development and performance in vitro with mtDNA content. In this instance, the association between mtDNA copy number and the capacity or level of ATP generation found in somatic cells is assumed to be similar. This assumption seemed to be validated in initial reports by the finding of low copy numbers in oocytes that failed to mature to metaphase II in vivo or fertilize in vitro [6, 17]. However, reported mtDNA contents range from the low ten thousand to over one million in normal-appearing MII oocytes [11, 18, 19], which can be problematic in determining threshold levels that may be stage-specific or, more importantly, developmentally relevant. For example, the threshold mtDNA copy number suggested to be normal for a competent MII human oocyte by Zeng et al. [12] is significantly higher than the level

proposed by Santos et al. [17] for competence. Further confounding the threshold issue is that mitochondrial mass and mtDNA copy number are often used interchangeably, and if each organelle contains one or two genomes at MII [20], the expected numerical complement of mitochondria implied by these findings might be in high hundreds of thousands [19]. However, while mitochondria are the most abundant organelles in the mature oocyte, fine structural images [14] and analysis of living cells with mitochondria-specific fluorescent probes [21] suggest a normal complement in the tens of thousands. Indeed, if mtDNA copy numbers at the high end of this range correlated with complement size, it would be evident at the fine structural level by virtue of a mitochondrial density that would occupy virtually the entire cytoplasm, which has not been the case in reported fine structural studies.

The simplest explanation that reconciles differences in mtDNA content and mitochondrial mass is that multiple genomes exist in each organelle, especially at MII, when most measurements have been made. This interpretation has been supported by counting individual mitochondria in each cell of fully expanded blastocyst-stage mouse and human embryos and in trophectodermal outgrowths of mouse embryos, with different mitochondria-specific fluorescent probes that target different mitochondrial sites or properties [21]. The assumption upon which these studies were based is that while mtDNA levels may fluctuate during oogenesis and preimplantation embryogenesis (see below), in aggregate, the relative number of mitochondria present in the periimplantation-stage blastocyst should reflect the complement present in the MII oocyte because mitochondrial replication begins after implantation [22], and to date, fine structural studies of normally progressing preimplantation-stage embryos show no degenerate forms or indications of mitochondrial replication [15]. Results derived from different mitochondria-specific fluorescent probes indicate that the 100-cell human blastocyst (ICM and trophectoderm) contains approximately 18,000–25,000 mitochondria. Previous electron microscopic analyses of normal-appearing MII human oocytes suggested that a mitochondrial complement around 25 K may be normal; however, mtDNA copy number derived from sibling oocytes detected genomic copy numbers between ~110,000 and ~240,000 [23].

Recent studies of mtDNA content during porcine oocyte maturation may clarify the apparent numerical discrepancy between mitochondrial complement size and mtDNA copy number. Spikings et al. [24] reported that similar to the situation in other species, a significant burst of mtDNA replication occurs during preovulatory maturation in the porcine, which results in an increase in genomic content that is several fold higher than levels measured at the germinal vesicle stage. However, such an increase is not observed in rat oocytes that matured in vitro [25], suggesting that the stage-specific upregulation of mtDNA synthesis may operate through signals transmitted to the immature oocyte at the outset of maturation through cumulus and coronal cells, which, in turn, may be responding to signal transduction cascades initiated by LH.

The above findings suggest that each mitochondrion in a normal-appearing MII-stage human oocyte likely contains multiple genomes which, if confirmed by additional studies, would go a long way in reconciling apparent differences between organelle mass and mtDNA content. The importance of reconciling mitochondrial mass and mtDNA content is not only to clarify apparent confusions in the literature but rather also to have a firmer basis for relating mitochondrial properties in the human oocyte and early embryo with competence. Low mtDNA copy numbers have been a suggested etiology of maturation and fertilization failure for human oocytes and for poor embryo performance in vitro [12, 26]. A better understanding of the extent to which fertilization or early embryo failure is related to an acute increase in mtDNA content during the terminal stages of oogenesis (i.e., preovulatory maturation), rather than to an actual organelle deficiency, could point to regulatory defects in the stage-related expansion of mtDNA. Confirmation for the human could make the signaling pathway for mtDNA expansion a focus of study for competence rather than the apparent endpoint, mtDNA content. In this regard, a potentially important finding of Spikings et al. [24] may have implications for the human: during cleavage, approximately 80% of the elevated mtDNA content measured in the mature oocyte was no longer detectable, suggesting that degradation of mitochondrial genomes (but not mitochondria) is a normal process during early development.

What could be the developmental significance of a process that rapidly increases the number of mitochondrial genomes during preovulatory maturation only to degrade them after fertilization? Given the comparatively undeveloped structure of oocyte mitochondria, a transient increase in mtDNA could alter the dynamic relationship between ATP supply and demand during certain stages of oogenesis and early embryogenesis when energy demands may be needed to support cytoplasmic remodeling, circulation, chromosomal segregation, and polar body formation [3]. In this regard, species-specific differences may exist in how differential energy demands may be supplied during the preovulatory and early embryonic stages. In the mouse, for example, spatial changes in mitochondrial density appear to be satisfied by the active translocation of mitochondria to the perinuclear region beginning around germinal vesicle breakdown [27, 28]. Van Blerkom and Runner [27] first proposed that active translocation in the mouse oocyte may be an adaptive mechanism that can elevate ambient levels of ATP in specific areas of the cytoplasm where demand is transiently higher. As discussed below, an increase in the magnitude of $\Delta\Psi_m$ or mtDNA copy number may be other strategies to achieve a similar end in species where significant cytoplasmic remodeling and redistribution of mitochondria are not apparent, such as in the human oocyte.

In certain instances, upregulation of mtDNA replication has been generally considered a compensatory mechanism to increase ATP production under conditions of reduced respiratory function resulting from the acute or chronic effects of toxic insults such as excessive superoxide production. A

similar phenomenon of increased mtDNA content has also been reported for sperm and oocytes. May-Panloup et al. [10] reported that the mtDNA content of human sperm with abnormalities known to compromise fertility was significantly higher than in normospermic samples. This was confirmed by Song and Lewis [29] for asthenozoospermic individuals, who also showed that the loss of DNA integrity was higher in affected men, which most was likely due to fragmentation and guanosine oxidation (8oxyG) resulting from excessive levels of mitochondrial superoxide production. In this instance, the increase in average mtDNA content was suggested to compensate for mtDNA mutations or fragmentation that may affect the electron transport chain and therefore respiratory (ATP generation) capacity. If an increase in mtDNA copy number occurs when mature sperm are in the ejaculatory pathway, potential upregulation of ATP production would not reverse structural alterations in plasma membrane integrity and function induced by lipid peroxidation, nor would it correct nascent mutations or defects in mtDNA integrity. The notion that increased mtDNA copy number may be a natural compensatory mechanism would seem to be contradicted by the clinical finding that even if such a process occurs, the sperm are still functionally compromised with respect to motility, and the affected men are still classified as infertile or subfertile.

Wang et al. [30] reported that fully grown MII oocytes obtained from streptozotocin (STZ)-induced diabetic mice show alterations in mitochondrial fine structure such as rupture of the outer membrane, changes in internal membrane organization, and organelle swelling. These defects are consistent with reduced mitochondrial function resulting from respiratory chain defects which, depending upon extent, could activate the mitochondria-dependent apoptotic pathway. However, against a background of mitochondrial alteration and damage, the mtDNA content of diabetic oocytes measured by quantitative real-time PCR was significantly higher than levels in untreated controls [31]. Similar to what may be an adaptive survival mechanism in other cells, increased mtDNA content was postulated by these investigators to be a compensatory mechanism to maintain ATP at levels required to support oocyte maturation and early development in the presence of respiratory chain dysfunction. However, a compensatory explanation does not account for the pronounced delay in meiotic maturation to MII observed in these mouse oocytes. In this regard, reduced mitochondrial function associated with disorders in the electron transport chain and abnormalities in stage-specific translocation and spatial remodeling of mitochondria, which have been proposed to focally balance ATP supply and demand during mouse oocyte maturation [27, 32], were suggested by Wang and Moley [31] to contribute to meiotic spindle malformations and errors in chromosomal segregation during oocyte maturation in the diabetic mouse model. The transmission of structurally or functionally compromised mitochondria would likely have toxic effects on development during the preimplantation stages that cannot be relieved by increasing mtDNA copies. Since a burst of mtDNA replication appears to be a normal feature of preovulatory maturation [24], the extent to which, if any, the mtDNA content in diabetic oocytes reflects this process, or is substantially different from levels detected in unaffected oocytes, remains to be determined. A similar issue concerns whether levels of mtDNA degradation during cleavage in STZ diabetic mice differ from controls. It seems unlikely that mitochondria in the oocytes of diabetic women are functionally compromised, as this disease is primarily associated with defects in follicular growth, ovulation, and maintenance of gestation, rather than disorders in oocyte maturation, fertilization, and early embryonic development. However, the assumption that mitochondrial function is normal in this instance remains to be investigated at the fine structural, biochemical, and mtDNA levels.

The notion that an inherent mechanism that upregulates mtDNA replication in order to compensate for bioenergetic deficiencies associated with sublethal mitochondrial dysfunction may be particularly relevant for women of advanced reproductive age. Maternal age is the foremost factor associated with the probability of natural cycle pregnancy and gestation to term birth, and it is no different with respect to outcome in assisted reproduction. A high proportion of oocytes obtained for IVF after ovarian hyperstimulation and ovulation induction in women of advanced maternal age are immature or, if mature, have a high probability of being aneuploid. If fertilized, the resulting embryos often arrest or develop abnormally during the preimplantation stages and are more likely to undergo demise after implantation than is the case for younger women. The possibility that mitochondrial mutations resulting in respiratory dysfunction may contribute to an age-related reduction in fertility and fecundity first received support from the studies of mtDNA by Keefe et al. [33], who reported that the frequency of a common mitochondrial deletion, the 4977 bp deletion (corresponding to nucleotide pairs 8482–13,460), was increased in the oocytes of older women. This common deletion also occurs in rhesus macaque oocytes and has been suggested to contribute to impaired mitochondrial ATP production [34]. Whether the relative size of the deletion, which is larger in the rhesus (5704 bp) than human (4977 bp), is related to the extent of detectable respiratory dysfunction is unknown. However, Muller-Hocker et al. [16] did not detect an increased frequency of either point mutations or the 4977 bp deletion in women of advanced maternal age (>40). These investigators did report fine structural morphometric results that indicated a significant increase in mitochondrial density occurred in older oocytes and that the diameter of mitochondria was also larger than measured in oocyte mitochondria of younger women. While the increase in mitochondria diameters may be due to slight swelling resulting from a $\Delta\Psi_m$ that is insufficient to maintain normal volume homeostasis [2, 35], their findings also showed no age-related functional defects in respiratory chain enzymes. Whether an increase in mitochondrial numbers and changes in organelle diameters indicate a compensatory mechanism to increase ATP production in the oocytes of women of advanced maternal age, as suggested by Muller-Hocker et al. [16], remains to be con-

firmed. However, unlike the upregulation of mtDNA content in functionally compromised sperm, a similar compensatory mechanism does not appear to occur in "older" human oocytes [36].

Correlating oocyte and embryo developmental ability with levels of mtDNA copy number, organelle complement, and bioenergetic capacity has been the basis of recent mitochondrial studies in animal models such as the bovine [37–39], pig [9], mouse [27], and human [12, 17]. For the human, a determination of threshold levels for each of these mitochondrial parameters has been suggested to represent possible analytic tools that could be used to diagnose fertilization or developmental failure. However, establishing a cause–effect relationship with regard to developmental competence may be problematic and more apparent than real. With respect to stage-specific bioenergetic thresholds, Van Blerkom et al. [4] reported that experimentally reducing net cytoplasmic ATP contents in the mouse by approximately 50% did not inhibit maturation from the germinal vesicle (GV) to MII stages, and while these treated oocytes were fertilizable in vitro, a high proportion of embryos arrested shortly after fertilization and none progressed to the blastocyst. Presumably, postfertilization developmental arrest was associated with irreversible mitochondrial damage with adverse downstream consequences. In contrast, Wai et al. [40] showed that mouse oocytes with as few as 4000 mtDNA copies were fertilizable and capable of developing to the blastocyst stage but died shortly after implantation. They concluded that (1) a threshold level of 40,000–50,000 mtDNA copies in the MII mouse oocyte was required to support development during the early postimplantation period and (2) high copy numbers in the mature oocyte were necessary in order to distribute mitochondria and mtDNA to the cells of the early implanting embryo prior to the initiation of mtDNA replication and organelle biogenesis. For the bovine, Chiaratti and Meirelles [39] reported no quantitative difference in mtDNA content between competent and incompetent embryos. However, in an elegant experiment, these authors removed ~64% of the mitochondria from MII oocytes after compartmentalizing these organelles to one pole of the oocyte by centrifugation. They found that oocytes, depleted of mitochondria by this extent, were fertilizable and competent to develop to the blastocyst stage. For these blastocysts, mtDNA contents were similar to nonmanipulated controls. The compensatory strategy employed by mitochondrially depleted embryos to restore normal mtDNA levels by the blastocyst stage is to upregulate the expression of TFAM and NRF1, two critical genes in mitochondrial function that control mtDNA replication and transcription, respectively. Chiaratti and Meirelles [39] reached two important conclusions for preimplantation development in the bovine: (1) an intrinsic mechanism exists in the early embryo to provide a threshold mtDNA content required for blastocyst formation, and (2) competent embryos can regulate mtDNA content regardless of copy numbers present at MII. The degradation of mtDNA detected during the cleavage stages [24] may be part of a self-regulatory mechanism to maintain a threshold mtDNA content required to support postimplantation development prior to mtDNA replication and mitochondrial biogenesis [40]. Because of the high degree of similarity between mammals with respect to the developmental biology of oocyte maturation, fertilization, and preimplantation embryogenesis, the above strategies for establishing a developmental threshold for mtDNA copy number may also apply to the human and, if confirmed, could indicate that the developmental consequences of a bioenergetic deficit may not be evident at MII or during the early stages of embryogenesis. However, testing this supposition experimentally using the same inhibitor treatments or invasive manipulations [39] to downregulate mitochondrial metabolism or reduce mitochondrial complement would require IVF and embryo culture. It is doubtful that such experiments would be considered acceptable or indeed ethical.

Although the above findings demonstrate molecular strategies used by the oocyte and early embryo to up- or downregulate mtDNA copy numbers, it is unclear how levels that may be below or in excess of a postimplantation threshold are recognized at the cellular level. As noted above, reported mtDNA contents between MII human oocytes in the same cohort can differ by over an order of magnitude, and similar differences have been reported for other species [38]. While speculative, one possibility may be related to the redox state of the cytoplasm and the influence mitochondria have on redox homeostasis, which in turn can regulate the activity of redox-dependent signaling pathways and other redox-sensitive regulatory factors (e.g., transcription factor, see below). The extent to which, if any, the redox state of the ooplasm or embryo cytoplasm can be related to mtDNA copy number and stage-specific mtDNA expansion or degradation warrants investigation.

76.3 Roles of Mitochondrial Reorganization During Early Development

Oncosis is a survival strategy that can be employed by some cells in order to adapt to transient reductions in ATP generation, such as during ischemic episodes [41]. In these instances, cytoplasmic remodeling results in mitochondrial translocation to the center of the cell, usually around the nucleus, and to balance ATP demand with reduced bioenergetic capacity, portions of the cortical cytoplasm are extruded as blebs that are largely devoid of mitochondria. The extruded cytoplasm remains connected to the underlying cell by cytoplasmic bridges. If normoxic conditions return and ATP levels rise, mitochondria disperse and the cytoplasmic extrusions are resorbed; if the restoration of normoxic conditions is within a cell type-specific tolerance, survival is indicated by the restoration of normal function. This novel mechanism of adaptation to transient anoxia or severe hypoxia has been suggested to operate in cleavage-stage human embryos that exhibit very similar cellular responses and characteristics [42]. One of the more remarkable features of human embryo performance during the early cleavage

stages in vitro are instances of fragment "disappearance" during subsequent culture, resulting in embryos that appear largely morphologically normal and stage-appropriate [23, 43]. This phenomenon has been observed by time lapse, even in embryos with fragmentation levels classified as high grade, where resorption restored stage-appropriate morphology and the affected cell(s) underwent division [42].

Light microscopic [44] and fine structural analyses [42] offer a possible explanation for the restoration of apparently normal cell function—the spherical fragments occur in columns that are interconnected to one and another and to the underlying cell by cytoplasmic bridges. The fragments contain few mitochondria that are mostly high potential and derived from the subplasmalemmal cytoplasm [45]. Van Blerkom et al. [42] suggested that abnormal patterns of cytoplasmic remodeling, possibly resulting from corresponding disorders in cytoskeletal organization, could locally reduce mitochondrial density in the pericortical cytoplasm in some cleavage-stage blastomeres. In contrast, remodeling does not appear to influence mitochondria in the subplasmalemmal cytoplasm, as discussed below. The compartmentalization of this cytoplasm into columns of interconnected extrusions (blebs) may be a local response to a focal ATP deficit. Restoration of a more normal distribution of mitochondria, especially in the cortical cytoplasm, was suggested to relieve the local bioenergetic deficit and return normal cell function, which is the situation when the oncosis-inducing stress is relieved. The persistence of fragments on cells that underwent a significant reduction in volume was considered to result from the failure of mitochondria to redistribute, and in these instances, high-density mitochondrial aggregates are observed in more central regions of the cytoplasm.

Whether by stage-specific up- or downregulation (degradation) of mtDNA content, active mitochondrial translocation, or perhaps an oncotic-like mechanism, adaptive strategies exist in cells to survive short-term ATP deficits and balance regional changes in the demand–supply equilibrium, and these mechanisms may also be employed by human oocytes and early embryos. The utilization of adaptive strategies is likely embryo-specific because within cohorts cultured in the same environment, not all are affected. This long-standing and consistent observation in clinical IVF demonstrates that at the blastomere level, the normality of development can differ from the embryo as a whole, which can develop progressively in the presence of cells that appear developmentally compromised owing to unique intracellular conditions, physiology, or genetics. At present, clinical and experimental findings from human IVF indicate the importance of mitochondrial function as a primary driver of normal development, although threshold levels of ATP generation and their relationship to mtDNA copy number remain unclear. This is not surprising considering that from the earliest classes one takes in biology, mitochondria are defined as the "powerhouses" of the cell, and this descriptor is still used in many scientific publications. However, what has emerged from studies of mitochondrial function in mammalian oocytes and nascent embryos is that how "power" is distributed within the cell and can be adjusted to meet local changes in demand is as important in understanding cell function during early development as is the power source [3]. Typically, studies of the bioenergetic state of the human oocyte and early human embryo have focused on ATP content at a particular stage. For example, several studies indicate that an ATP content around 1.8 pM may be a steady-state level consistent with competence for the oocyte competence and early human embryo [4, 12]. However, the values obtained represent the net cytoplasmic content at the time of measurement because rates of ATP turnover are rarely determined, and developmental competence is assumed because ATP quantitation requires cell lysis. Therefore, conclusions about developmentally significant threshold levels represent comparative values derived from oocytes that fail to mature in vivo or in vitro, or fertilize in vitro, or from embryos that arrest cell division or show apparent common performance and developmental abnormalities during cleavage, such as grossly unequal cell divisions, high-grade fragmentation, or blastomere multinucleation. If adaptive mechanisms are employed during early development to overcome bioenergetic deficits, they are apparently insufficient in a compensatory context in these instances.

76.4 Mitochondrial Inheritance After Fertilization

Although ATP measurements appear to provide a general impression of bioenergetic states that could be of clinical value if related to stage-specific threshold levels, performance defects such as those noted above cannot be assumed, a priori, to have such an etiology. For example, measurements of ATP levels in human embryos that arrest development at the pronuclear to 8-cell stage do not consistently or necessarily have ATP contents that differ significantly from their normally progressing counterparts (e.g., dispermic fertilization) or from normally fertilized siblings cryopreserved at the pronuclear stage and cultured through the cleavage stages after thawing (unpublished). In contrast, the failure to develop into a normally compacted morula that can initiate cavitation, or the inability of a cavitated embryo to progress to the expanded blastocyst stage, which is an energy-intensive process, is more likely to have an origin associated with mitochondrial structural or functional defects [15, 16]. While quantitation of the ATP content(s) of whole embryos may not be informative in a diagnostic sense, or capable of identifying a specific cause of embryo arrest or developmental abnormality, semi-quantitative assessments of mitochondrial mass in individual blastomeres may have clinical value in this regard. Van Blerkom et al. [43] showed that quantitative values for bioenergetic state and comparative mitochondrial mass could be assessed in the same blastomere(s) with highly sensitive organelle-specific fluorescent probes used for the latter. Examination of mitochondrial fluorescence alone in intact 2- and 4-cell stage embryos showed disproportionate mitochondrial segregation with subsequent performance

in vitro related to stage (2–8 cell) and extent. These investigators suggested that the origin of this pattern of disproportionate mitochondrial inheritance was related to the symmetry of peripronuclear mitochondrial aggregation at the 1-cell stage. For normally developing fresh and thawed embryos between the 2- and 16-cell stages, the relative intensity of mitochondrial fluorescence and net cytoplasmic ATP content were largely similar between blastomeres. Subsequent studies have shown that blastomere-specific differences in the relative intensity of mitochondrial fluorescence are a comparatively common theme for embryos that developed no further than the 4- or 8-cell stage, with blastomere-specific differences in relative intensity up to 80% observed (unpublished). These findings suggest that a focus on noninvasive methods of assessing mitochondrial mass at the blastomere level could provide important clues related to competence for the entire embryo.

76.5 Functional Compartmentalization of Mitochondrial Activity

Several lines of investigation indicate that intracellular mechanisms that readjust ATP supply and demand within the oocyte and early blastomere may be a critical determinant of the normality of development. Local changes in ATP demand can be met by transient changes in mitochondrial distribution using active (cytoskeletal elements such as microtubules) and passive (intracellular circulation) mechanisms that affect organelle density and ambient bioenergetic state [2, 3, 46]. An increased potential across the inner mitochondrial membrane is also associated with higher levels of ATP generation, and in some cultured cell lines, changes in $\Delta\Psi_m$ have been reported to be location dependent. In certain instances, high-potential mitochondria are localized to the cell margins where the plasma membrane is motile, with lower potential forms in the interior, especially around the nucleus [47]. These investigators reported that subplasmalemmal mitochondria shift from high to low potential at zones of intercellular contact, but when contact is interrupted, return to high potential where the cell margins are free. In these cells, changes in ATP demand required to support plasma membrane dynamics at the free margins of a cell appear to be met locally by increasing $\Delta\Psi_m$ rather than by mitochondrial translocation to increase organelle density. In other cells, local changes in demand are accommodated by mitochondrial aggregation, which by virtue of altering ambient cytoplasmic redox state (see below) can increase focal levels of respiration in the aggregate [46]. A similar focal change in $\Delta\Psi_m$ in subplasmalemmal mitochondria has been reported for early cleavage-stage mouse embryos [48]. At the zone of gap-junction-mediated contact between blastomeres, the corresponding mitochondria are low potential but become high potential when the cells are separated. Mitochondria at the free margins are high potential. When repositioned 180° at the two-cell stage, such that the formerly free margins are in contact, high-potential shifts to low potential, and vice versa.

Mechanisms of mitochondrial redistribution, translocation, and aggregation detected in cultured cells also occur during early development. In some species, such as the mouse [27, 28] and hamster [49], microtubule-mediated mitochondrial translocation [32] during oocyte maturation and early postfertilization development results in the formation of a relatively dense sphere of mitochondria around the developing nuclear region of the oocyte and juxtaposed pronuclei of the 1-cell embryo. Van Blerkom and Runner [27] first proposed this pattern of cytoplasmic remodeling readjusts ATP supply to accommodate higher ambient energy demands associated with the evolving nuclear region in both oocyte and nascent embryo. In the maturing mouse and human oocyte and pronuclear stage embryo, mitochondria in the subplasmalemmal cytoplasm have an apparent $\Delta\Psi_m$ that is significantly higher than exhibited by the vast majority of mitochondria within the cell [48]. It is worth mentioning that mitochondria which are actively translocated to the perinuclear region during oocyte maturation in the mouse are low potential and that the high-potential forms in the subplasmalemmal region do not participate in this redistribution [48] and remain spatially stable and at a comparatively high potential from the oocyte through the cleavage stages [50].

Although the subplasmalemmal domain contains less than ~3% of the complement of mitochondria in the mature oocyte, their loss by minor fragmentation from this domain is apparently irreversible, and depending upon the degree of loss, adverse developmental effects including delayed or arrested cell division have been reported [50]. Similarly, failure to assume a high $\Delta\Psi_m$ at MII, or a domain of high-potential forms that is scant and largely discontinuous, has been associated with sperm penetration failure in the human after conventional IVF [48, 51]. It has been suggested that high potential and a subplasmalemmal localization may provide higher ambient concentrations of ATP to support ATP-driven plasma membrane activities that include sperm penetration and migration of the incorporated sperm nucleus [3, 52]. Reversibly reducing $\Delta\Psi_m$ in this domain was inconsistent with penetration until high potential was restored [50].

76.6 Intracellular Free Calcium and Mitochondrial Activity

The above findings suggest that mitochondrial potential, location, and density are mechanisms by which ATP may be differentially generated, distributed, and utilized within the cytoplasm of the oocyte and early embryo, and defects in each element of this energy management system could affect competence depending on stage and extent. As noted above, the absence of high $\Delta\Psi_m$ in the subplasmalemmal domain at MII is associated with penetration failure for both fresh [48, 50] and thawed human oocytes [51]. Local changes in the

concentration of intracellular free calcium is another critical element in the regulation of mitochondrial ATP production and, for the human, may be directly related to developmental abnormalities that can occur well beyond the preimplantation stages [53]. Mitochondria are excitable organelles [54] that respond to changes in ambient free calcium levels by releasing or sequestering calcium, and the level of response can be related to the magnitude of $\Delta\Psi_m$. Mitochondria respond to calcium released from intracellular stores, such as the smooth-surfaced endoplasmic reticulum (sER), through the calcium-induced calcium release pathway (CIRC) [55, 56] and from calcium released by mitochondria themselves through the mitochondrial calcium-induced calcium release (mCIRC) [57]. It has long been known that calcium is a regulator of mitochondrial ATP synthesis [58]. Dumollard et al. [59] demonstrated that by experimentally manipulating calcium release from sSER, ambient calcium levels and mitochondrial activity were tightly coupled in a regulatory manner that could up- or downregulate levels of respiration in the newly fertilized mouse oocyte. The benefit for the oocyte or early embryo of such tight coupling is that local, stage-specific ATP demands can be met without involving the entire mitochondrial complement, which has the potential of increasing levels of ROS (superoxide). In the mouse and human oocyte and early embryo, fine structural analyses show cisternae of the sER in contact with mitochondria [14], especially in the pericortical/subplasmalemmal cytoplasm where of mitochondria surround sER aggregates [23, 48]. Van Blerkom et al. [48, 60] suggested that the fertilization-induced influx of calcium may increase mitochondrial activity in the subplasmalemmal domain and possibly initiate mCIRC along the circumference of the oocyte, which could locally assist in the cortical granule exocytosis and other calcium-dependent activities associated with sperm penetration. The validity of this notion remains to be determined experimentally.

Little is known about spontaneous abnormalities in calcium release at the earliest stage of human fertilization and whether defects in calcium signaling [61] or levels could have downstream effects on mitochondrial activity or the normality of embryogenesis. Typically, studies of changes in cytoplasmic free calcium involve preloading oocytes with a fluorescent calcium reporter such as Fluo-4 AM [51, 60] followed by activation with a calcium ionophore (e.g., A23178; [3, 56]) or insemination by intracytoplasmic sperm injection (ICSI) [62, 63]. The normal pattern of free calcium fluorescence observed by scanning laser confocal microscopy after human oocyte activation is shown in Fig. 76.1a–d. Shortly after ionophore exposure, the relative intensity of fluorescence increases throughout the cytoplasm (Fig. 76.1b, c) before decaying to levels (Fig. 76.1d) that existed in reporter-labeled oocytes prior to activation (Fig. 76.1a). In contrast, within the same cohort(s) of MII oocytes activated 2–4 h after follicular aspiration and hyaluronidase-mediated corona and cumulus cell denudation, the rise in fluorescent intensity in some oocytes was regional (asterisk, Fig. 76.1e–f) rather than a uniform. In most instances, the intensity of calcium fluorescence declined to background levels at rates similar to those observed in oocytes with a uniform rise. The oocytes shown in Fig. 76.1e, h are representative examples of a pattern of cytosolic calcium fluorescence that was an exception. Fluo-4 AM fluorescence was detectable for at least 2 h after levels returned to background in similarly treated oocytes, such as those shown in Fig. 76.1d, g. The average net ATP content measured 4 h after activation in oocytes with these aberrant patterns of free calcium fluorescence was significantly higher by ~30–50% (3.3 pM, ±0.6 pM, n = 23) than normal for MII human oocytes [4] that exhibited a uniform calcium rise (1.8 pM, ±0.3 pM, n = 15). A very similar phenomenon occurs in MII human oocytes cryopreserved by programmed (slow) cooling which, after thawing, are preloaded with this calcium probe and ionophore activated. Preliminary findings indicate that while <4% of fresh oocytes display this abnormal pattern (1/27), distinct regions of intense fluorescence occurred in approximately 42% (13/31) of oocytes that were of similar (normal) appearance at cryopreservation and activated 2–4 h after thawing (similar to Fig. 76.1e–h). However, ATP levels measured at 4 h after activation were not significantly different from levels measured in similar oocytes prior to cryopreservation [51].

The above findings suggest the possibility that oocyte-specific defects in sER function may be associated with the slow cooling cryopreservation, but freezing per se does not appear to affect global cytoplasmic ATP content after thawing. In this regard, the unusual patterns of calcium rise and the corresponding increase in net cytoplasmic ATP content observed in fresh oocytes could support the notion of tight metabolic coupling between sER-derived calcium and mitochondrial ATP production discussed above. The absence of a similar increase in ATP content in thawed oocytes could indicate cryopreservation-induced damage to mitochondria that makes them less responsive to calcium at the 4-h time point or an abnormal increase in ATP demand that requires higher levels of production that is not evident when total net cytoplasm ATP content is measured. Whether oocyte cryopreservation by slow cooling irreversibly alters mitochondrial organization at the fine structural level is unclear. While some reports have described alterations that could compromise function [64], others have not [65, 66], and in our experience, mitochondria in MII human oocytes cryopreserved by slow cooling that remain intact during the first hour after thawing appear unchanged from their fresh counterparts (unpublished). This is not to say that mitochondria are not damaged during slow cooling cryopreservation, but the damage may be reversible or occur at a level that does not alter respiratory capacity reflected by net cytoplasmic ATP content. Whether calcium-dependent signaling pathways are perturbed by this method of cryopreservation is unknown, but cellular bioenergetic state seems unperturbed.

The most compelling evidence to date of an association between atypical sER calcium release and ATP production occurs in ionophore-activated MII human oocytes that contain a single, large, centrally located disk-like inclusion composed of cisternae of the sER [67] (asterisk, Fig. 76.1i, k).

After preloading with the calcium reporter and exposure to A23187, activation is rapidly followed by an intense flare of fluorescence (2 min, asterisk, Fig. 76.1j) that coincides with the position of the inclusion. The intensity of fluorescence remains elevated for approximately 2–3 h (2 h, Fig. 76.1l), which is significantly longer than observed in unaffected oocytes [60]. Preliminary results indicate that net cytoplasmic ATP content measured at 1–4 h was ~2.0 to 2.5-fold higher than normal (3.3–4.6 pM) and remained elevated for at least 22 h before abruptly dropping to levels ≤50% of normal (0.6–0.8 pM).

The most remarkable characteristic of these oocytes was the behavior of the inclusion during prolonged culture as detected by time lapse. Figure 76.2 shows selected images at the indicated times after activation of an MII oocyte with a large sER inclusion. The arrows show the direction of movement of the disk, with the black arrows denoting movement detectable within the plane of focus and the white arrows denoting the direction of movement when the disk moves out of and returns to the plane of focus. Over this 22-h period, the disk moves throughout the cytoplasm going out of and back into the plane of focus, often oscillating back and forth within a 20–35 micron region. The calculated rate of movement during this time was relatively constant at ~1.0–1.5 μm/min. Exposure of oocytes to inhibitors of oxidative phosphorylation [60] caused this motion to cease within minutes, but resumed when the inhibition was relieved, if the duration of inhibitor did not exceed ~30 min. A close inspection of these representative images shows that the movement of the disk (indicated by a white asterisk) is accompanied by

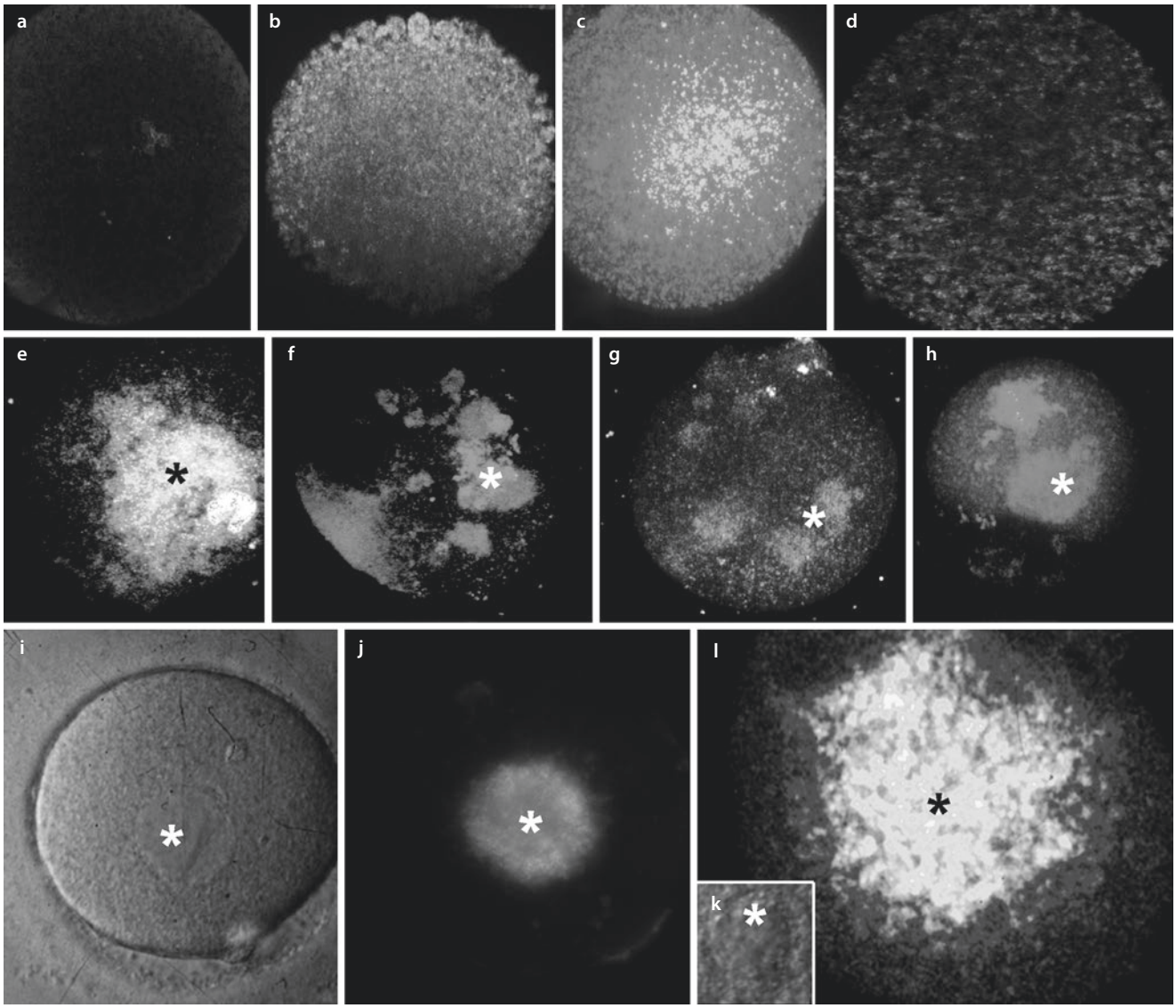

Fig. 76.1 Normal **a**–**d** and abnormal **e**–**h** patterns of increased levels of intracellular free calcium (*asterisk*) after ionophore activation of MII human oocytes. **j**, **l** Show a highly intense and prolonged flare of fluorescence, detected by a fluorescent calcium reporter, which corresponds to calcium released from a single, large aggregate of sER cisternae (*asterisk*, **i**, **k**). This sER aggregate defines an abnormal human oocyte phenotype, and its occurrence during IVF has been associated with unusually high levels of ATP generation and genomic imprinting disorders in newborns

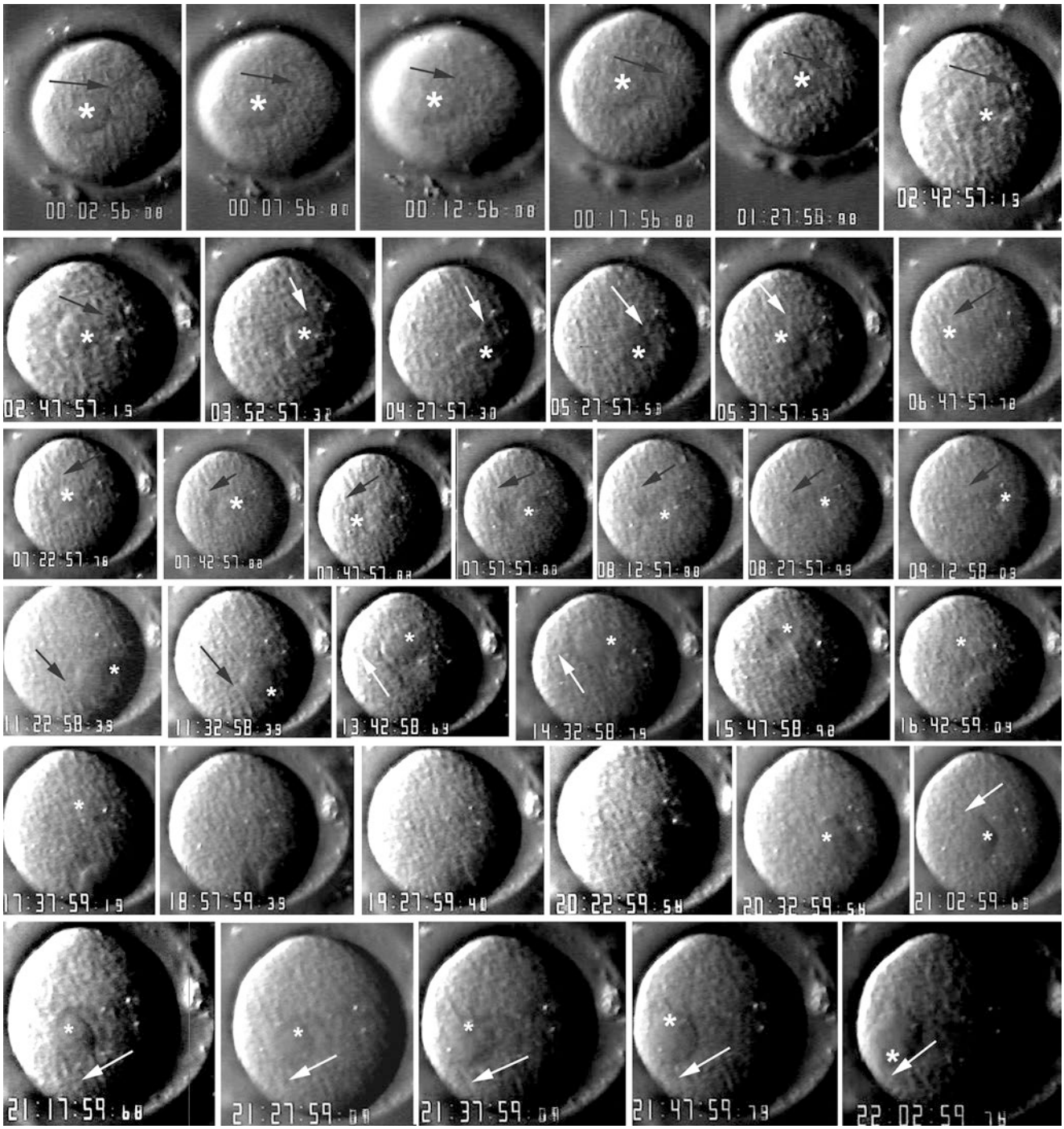

Fig. 76.2 Representative time-lapse images showing the movement of a single, larger sER inclusion (*asterisk*) in an MII human oocyte over a 22-h period. The *arrows* indicate direction at different times (indicated in *lower left*) as the inclusions move out of and into the plane of focus. This usual and persistent behavior is suggested to be supported by an enhanced cytoplasmic bioenergetic state associated with the maintenance of unusually high levels of mitochondrial ATP synthesis

corresponding dynamic changes in the cytoplasm immediately in front of and behind this structure (e.g., arrow, 17 min 56 s; black asterisks, 21 h 17 min 59 s to 21 h 47 min 59 s).

Time-lapse imaging has shown that after fertilization in the human, cytoplasmic motility increases in general and within the pericortical cytoplasm in particular [68]. However, a similar intensity and persistence of movement, such as displayed by these inclusions, have not been reported. It is tempting to speculate that the abnormal pattern of calcium release and the persistence of calcium-driven fluorescence affect the fluidity of the cytoplasm, perhaps by altering calcium-dependent structural elements responsible for cytoplasmic organization and circulation [2]. Together with an unusually elevated bioenergetic state, the condition of the cytoplasm may become permissive for and enhance dynamic movements of cytoplasmic components. In this regard, the

behavior of the sER inclusion may be governed and directed by mechanisms similar to those that promote the movement of the sperm nucleus after penetration and pronuclear migration during the 1-cell stage [52]. In these instances, pronuclear migration is associated with normal levels of calcium release that are periodic (so-called calcium transients) and begins with an initial increase at the earliest stage of the fertilization process. Although likely, the extent to which defects in these transients (occurrence or amplitude) are associated with fertilization failures in human IVF where the sperm nucleus remains (unexpanded) in the pericortical cytoplasm [69], or where pronuclear migration and juxtaposition do not occur [52], remains to be determined. However, whether the abrupt cessation of the SER disk movement described above reflects reactive oxygen toxicity to mitochondria resulting from the generation of superoxide in excess of the intrinsic antioxidant capacities (e.g., mitochondrial superoxide dismutase) is one possibility under investigation.

These findings indicate that abnormalities in calcium release may have downstream developmental effects if calcium-dependent signaling pathways or processes are perturbed. Two reports, one experimental and the other clinical, strongly support this possibility. The studies of Ozil and Huneau [53] showed that the developmental consequences of increasing the level of calcium released after ionophore activation of rabbit oocytes were not evident until organogenesis, where multiple system anomalies and defects resulted in fetal demise. It is important to emphasize that preimplantation development was normal. For the human, oocytes with a large SER aggregate such as shown in Fig. 76.1i, k are fertilizable by ICSI, but few embryos develop normally during the preimplantation stages and, if transferred, most cease to develop [70]. However, what is disturbing about some of the births from oocytes of this type is the occurrence of genomic imprinting disorders that have been suggested to be associated with aberrant calcium signaling at the outset of development [71]. Concerns about developmental normality and a high potential for imprinting disorders have resulted in the recent conclusion by a joint ESHRE/alpha panel of experts that human oocytes with SER inclusions, such as those described above, should never be inseminated [72].

76.7 Mitochondria and Signal Transduction Pathways

Similar to differentiated cells [73], a structural relationship between the SER and mitochondria [14, 48] in the oocyte and early likely includes regulatory functions other than calcium homeostasis and mitochondrial energetics. What is critical to understanding the extent to which abnormalities in calcium release at fertilization may affect human development is a determination of if, and how, the flow of regulatory information within the ooplasm may be perturbed and, for the early embryo, whether bidirectional information flow between the cytoplasm (e.g., transcription factors and activators) [74] and nucleus (e.g., mRNA) is altered. In this context, the identification of defects in signal transduction may provide fundamental insights into the origin of developmental competence for the oocyte and how it may be lost or compromised after fertilization. For the fresh oocyte, the occurrence of calcium release abnormalities appears to be a relatively low-frequency event, and the question remains why only some oocytes are affected. For thawed oocytes, however, the frequency is significantly higher, and the extent to which this abnormality is protocol related is unclear, and the results of fine structural analyses conflict with respect to cooling and freezing protocol. While some studies report no evident alterations in sER structure or organization [65], others have described apparent swelling of the cisternae and changes in spatial organization [75] that could contribute to the atypical response to ionophore activation shown here. While it is likely that an optimized protocol of oocyte cryopreservation that minimizes damage to or alteration of sER integrity and function will be forthcoming, for some oocytes, defects in sER function may be an inherent problem associated with fertilization failure or abnormal preimplantation embryogenesis. How these defects may influence mitochondrial function, cytoplasmic bioenergetic state, and local stage-specific free energy availability are the types of developmentally significant questions that, while currently unanswered, may be fundamental to understanding the cell biology of the human oocyte and early embryo.

76.8 Mitochondria and Cytoplasmic Redox State

An important regulatory influence of mitochondria in somatic cells is their effect on the redox state of the cytoplasm. The normal state of the cytoplasm is a reducing environment, and cells employ different mechanisms to maintain redox homeostasis in the presence of mitochondria-derived oxidative stress. The pathogenesis of certain diseases is based on the inability of these mechanisms to cope with oxidative stress, such as superoxide toxicity, and at the cellular level, an inability to counter a shift toward an oxidative environment can lead to signaling disorders and bioenergetic insufficiency leading to dysfunctions in cytoplasmic bioactivities, apoptosis, or pathological cell death. Mitochondrial redistribution and localization is one means by which location-dependent ATP supply and demand requirements can be met, and potentially higher levels of superoxide production that may occur with cytoplasmic mitochondrial aggregation or remodeling are normally addressed by mitochondrial and cytoplasmic antioxidants (e.g., superoxide dismutase and glutathione).

Location-related differences in mitochondrial density that alters cytoplasmic redox potential may also regulate redox-sensitive signaling pathways and in some species, such as the sea urchin, are essential for specification of the oral-aboral axis, which involves redox-dependent signaling path-

ways. The establishment of this axis in the fertilized egg is regulated by an asymmetrical mitochondrial distribution in the oocyte and in the early embryo, where a redox gradient is formed by virtue of differential mitochondrial density [76, 77]. These investigators reported that the portion of the sea urchin embryo that inherits the highest density of active mitochondria is strongly biased toward oral axis specification owing to the location-specific expression of the oral axis determining transcription factor, *nodal*. According to this model, a "transcription factor" gradient is established in the blastomeres of the early embryo in which the function of different redox-sensitive transcription factors, such as *nodal*, is dependent upon a redox threshold within the redox gradient established by differential mitochondrial density. In this context, the cytoplasm of the oocyte and early blastomeres may be functionally compartmentalized as a normal consequence of relative mitochondrial density.

A similar phenomenon, albeit on a much smaller scale, has been observed in differentiated somatic cells where mitochondria aggregate in different locations in order to increase ambient ATP availability to accommodate transient changes in local energy demand [46]. Spatial remodeling of mitochondria is a dynamic process and, in active cells, may undergo continuous redistribution. Changes in mitochondrial density may also alter local cytoplasmic redox state and, as a result, locally affect bioactivities with different redox-sensitive thresholds. This form of dynamic cytoplasmic mitochondrial reorganization was suggested by Aw [46] to be a type of microzonation in which local activities could be functionally compartmentalized within the cytoplasm in response to changes in cell activity or exogenous signals such as cell contact. Changes in mitochondrial density that alter the ambient redox state may also increase respiratory activity by the aggregated mitochondria if local pH is also reduced (by ATP hydrolysis), which Aw [46] suggests can increase the efficiency and rate of uptake of intermediately metabolites. Dynamic changes in $\Delta\Psi_m$ may be another example of mitochondrial microzonation. As described above, Diaz et al. [47] showed that the magnitude of $\Delta\Psi_m$ in mitochondria at the margins of cultured cells was cell contact dependent with a downregulation of this transmembrane potential occurring in areas of intercellular contact and communication. As previously noted, similar finding was reported for the cleavage-stage mouse embryo [48], where mitochondria in the subplasmalemmal cytoplasm corresponding to regions of intercellular contact and gap-junction-mediated intercellular communication were low potential while those beneath the free margins of the plasma membrane were high potential. In this study, experimental manipulations of early cleavage-stage embryos showed that the magnitude of $\Delta\Psi_m$ in these regions could be up- or downregulated as a function of the presence of or absence of intercellular communication and contact. It may be worthwhile to determine whether differences in redox state occur in these regions and, if confirmed, whether the absence of adequate cell contact or communication influences $\Delta\Psi_m$ and the normal functions of redox- and ROS-sensitive signaling (e.g., JAK-STAT) [78] in this region. This may be especially relevant in certain early cleavage-stage human embryos where the apparent absence of normal cell contact and communication appears to lead to aberrant development in vitro.

Findings from cultured cells and early embryos such as the sea urchin clearly demonstrate that in addition to ATP production, mitochondria have central regulatory roles in signal transduction through calcium and redox-sensitive pathways and, by creating microzones that may alter local physiology, can create cytoplasmic compartments with different functional characteristics. Evidence suggesting that similar cell biological activities occur in the mammalian oocyte and early embryo has come from the characterization of dynamic, stage-specific changes in mitochondrial distribution and from the detection of differential $\Delta\Psi_m$ that is location-based. Preovulatory cytoplasmic maturation of the oocytes of certain species, such as the mouse, involves stage-specific mitochondrial translocation to the perinuclear nuclear region [27, 28] that, in the mouse, is directed by microtubular arrays emanating from perinuclear microtubular organizing centers [32]. The formation of a relatively dense sphere of mitochondria around the developing nuclear region after germinal vesicle breakdown has been suggested to increase ambient ATP levels but may also alter the perinuclear redox state to promote formation of the first and second meiotic metaphase spindles, chromosomal segregation, and polar body abstriction [21]. Here too, the notion of thresholds may be developmentally significant as excessive or inadequate perinuclear aggregation may have coincident effects on bioenergetic and redox state that are inhibitory for maturation to MII [27, 79] or contribute to disorders in chromosomal segregation leading to aneuploidy [5, 80, 81]. In addition to the mouse [27], perinuclear aggregation has also been reported for the pronuclear and early cleavage stages in other mammals such as the pig, hamster, nonhuman primate, and the human [43, 82, 83]. Indeed, a pronounced peripronuclear accumulation of mitochondria occurs in the human, and asymmetries in distribution have been related to disproportionate mitochondrial inheritance during cleavage [43]. In contrast to the mouse oocyte, mitochondrial translocation to the developing nuclear region of the maturing human oocyte is less pronounced, perhaps owing to the absence of definitive microtubular organizing centers, especially in the perinuclear region. In addition, virtually all information concerning the cytoplasmic dynamics of human oocyte maturation has come from observations made in vitro with immature oocytes, most of which had failed to reinitiate meiosis or arrested meiosis prior to MII after ovulation induction. Reliance on in vitro matured oocytes for these studies is understandable because extracting oocytes at specific stages of preovulatory maturation after ovulation induction in stimulated or natural cycles (which would be preferable) is unlikely to be the type of experiment whose inherent value can be ethically justified. However, a limited number of fine structural images of human oocytes that were at the GV stage at aspiration, or remained so during culture in vitro, often show unusually high densities of perinuclear mitochondrial

aggregation [14]. Confirmation that an abnormal perinuclear redox state may be responsible for the failure of meiosis to resume or progress to MII would go a long way in providing new insights into how maturational and fertilization competence is established and, for the human, possible follicle-specific influences that promote or inhibit early development.

76.9 Mitochondrial Inner Membrane Potential and Functional Microzonation

The notions of microzonation and functional compartmentalization would be more compelling as developmentally significant aspects of oocyte maturation and early embryogenesis if they could be correlated with known abnormalities in cytoplasmic organization that have negative developmental consequences—the so-called cytoplasmic dysmorphisms described for human oocytes [67, 70]. At present, differential $\Delta\Psi_m$ appears to be one aspect of mitochondria that may support these notions [2, 21]. In the human and mouse MII oocyte, high-potential mitochondria are normally localized to the subplasmalemmal cytoplasm where they form a distinct circumferential domain detectable with $\Delta\Psi_m$-sensitive (potentiometric) fluorescent probes such as JC-1 [48, 84, 85]. In the human, the absence of high potential in this domain in fresh [48] and thawed MII-stage oocytes [51] has been suggested to be a proximal cause of penetration failure in conventional IVF. A similar phenomenon has been reported for the mouse where sperm penetration is reversibly inhibited by experimentally down- and upregulating $\Delta\Psi_m$, respectively, in this domain [86]. In the mouse and human, this domain is spatially stable in the oocyte and remains so during early cleavage, and in the human, spatial stability is indicated by the absence of high-potential forms after loss by minor fragmentation [50]. While their energetic contribution to net cytoplasmic ATP levels in the MII oocyte is negligible, which would be expected considering the domain contains <3% of the total mitochondrial complement, as noted above, their loss or reduced potential in this specific region appears to have important implications for development. The possibility that this extended domain (microzone) of mitochondria may have specialized functions during early development (functional compartmentalization) is suggested by the extent of loss, either naturally occurring or experimentally induced, on the subsequent ability of the affected blastomere(s) to participate in embryogenesis. The loss of high $\Delta\Psi_m$ organelles to a few small fragments had no discernable effect on embryogenesis, whereas loss from one or more regions that significantly diminished mitochondria from this domain, or where the distribution of high-potential mitochondria was scant or discontinuous, was associated with the failure of the affected blastomere to divide. However, loss to this extent is not necessarily lethal, as the affected blastomeres typically remained undivided as the remainder of the embryo progress through the preimplantation stages. At the fully expanded blastocyst stage, the presence of a cleavage-sized cell in the perivitelline space or less commonly the blastocyst cavity may originate from an early, nonlethal, loss of high-potential mitochondria from a single blastomere.

Wilding et al. [87] reported that clusters of high-potential mitochondria occurred within the cytoplasm of arrested/degenerating human blastomeres, but not in the subplasmalemmal cytoplasm. This phenotype has been rarely observed in our experience but could be associated with a premorbid state that may lead to apoptosis, as this cell death pathway generally involves an abrupt collapse of high $\Delta\Psi_m$ leading to the release of calcium and cytochrome c. In contrast, pathological cell death usually results from a persistently low $\Delta\Psi_m$ that corresponds to a bioenergetic state that is unable to meet the minimal ATP demands required to maintain cell function. In this regard, most unfertilized human oocytes will remain intact for days in culture, and changes in mitochondrial fine structure, such as loss of cristae and changes in matrix electron density that become apparent after day 4 or 5, are consistent with a significant drop in net ATP content and scant high potential in the subplasmalemmal domain [3]. Typically, these oocytes lyse on or after day 6 or 7, indicating that cell death is likely due to an energetic deficiency associated with necrosis rather than by the induction of apoptosis [45].

While there is compelling evidence that a subplasmalemmal domain of high-potential mitochondria exists in the mature human oocyte and cleavage-stage embryo and appears to be involved in sperm penetration, why perturbation of this microzone at the blastomere level has adverse consequences remains to be determined. One possibility suggests that higher ambient levels of ATP may be required to support dynamic membrane activities involving transporters, ion channels, and plasma membrane reorganization between the oocyte and embryonic stages. Another possibility that warrants investigation is that this domain is involved in signal transduction pathways and those that are redox- or ROS-sensitive in particular [3]. If confirmed, findings of this type may add a new dimension to our understanding of what competence entails during the earliest stages of human development. Likewise, they may provide novel diagnostic methods to assess subtle causes of fertilization failure, embryo arrest, and abnormal performance in vitro that may be related to maternal age or for some patients, to repeated negative outcomes in IVF cycles.

The role of mitochondria in the regulation of cytoplasmic redox state may be one that is unfamiliar to clinical practitioners, but is nevertheless a fundamental property that, as more information emerges, will likely become another factor to be recognized in the establishment of competence. Likewise, the relationship between mtDNA copy number at MII and whether numerical expansion occurs during the preovulatory stages may have important influences on mitochondrial activity in the oocyte and redox state and ROS/redox-sensitive signaling during fertilization and early embryogenesis. A similar activity may occur during the cleavage stages where high-potential mitochondria remain localized in the subplasmalemmal cytoplasm. It remains to be determined whether loss of these mitochondria to fragmentation, or failure to

resume high potential after thawing [51], impacts the local redox state or redox-sensitive signaling at the level of the plasma membrane and subplasmalemmal cytoplasm.

A potentially important finding demonstrating that cytoplasmic redox potential can be altered by exogenous factors comes from the recent study of Dumollard et al. [88], who reported that the normality of preimplantation embryogenesis in vitro was supported by a redox potential that involved cytosolic and mitochondrial metabolism of pyruvate and lactate—the former a cytosolic oxidant and mitochondrial reductant and the latter a strong cytosolic reductant. The potential developmental significance of these findings is that cytoplasmic redox potential can be altered by varying the concentration of intermediate metabolites normally found in embryo culture medium, such as pyruvate. In this context, seemingly minor modifications to culture medium that may change the oxidation/reduction equilibrium in a stage-dependent manner could be unrecognized factors that directly impact human embryo performance and viability during the preimplantation stages. Novel fluorescent probes that can assess intracellular redox state in living cells [89] may be sufficiently sensitive to detect local state changes that may be associated with stage-specific events such as fertilization and intercellular communication and possibly with modifications to medium composition or culture conditions. In this regard, it will be relevant to determine the extent to which, if any, metabolomic assessments of spent culture medium that have been proposed for clinical IVF [90] are detecting redox changes that could influence competence. What is particularly interesting about this possibility is that it could add redox homeostasis to the calculus of assessing outcome if differences are oocyte−/embryo-specific or cohort-wide and perhaps suggest novel treatments that can be implemented to target functional disorders that are redox (mitochondrial)-driven.

76.10 Summary and Future Prospects

While a considerable body of evidence supports a central role of mitochondria in early human development, questions other than those discussed above are worthy of mention. What is the normal number of mitochondria in an oocyte, and is there a threshold for competence? Often, mitochondrial mass and mtDNA copy number are used interchangeably, which can confound this issue as mtDNA copy numbers can vary widely, often by over an order of magnitude, between MII oocytes in the same cohort [2, 11, 18, 23]. Variations in mtDNA content between oocytes within the same and different cohorts may reflect differential levels of mtDNA expansion among mitochondria rather than numerical differences in mitochondrial complement. Resolution of this issue will require morphometric analyses, preferably with superfluous MII oocytes whose cytoplasm is characterized as normal [72]. A second question is whether mtDNA expansion occurs during preovulatory maturation, at what stage(s), and whether mtDNA degradation occurs during cleavage and how it may be regulated [24]. Whether the finding of lower mtDNA content found in rat oocytes that matured in vitro [25] pertains to the human needs to be determined if IVM is to become a standard practice in clinical IVF.

At present, while these issues are speculative, they do indicate the types of questions that may provide basic information about cell biology of the human oocyte during the terminal stages of development and at the earliest stages of embryogenesis. Against this background, the extent to which differential mitochondrial potential or activity may influence local redox state and, by extension, putative redox-sensitive or redox-dependent signaling pathways may be among the most relevant because the effects of altered signaling during embryogenesis in other developing systems, such as those described above, can be both immediate and downstream, and there is no reason to expect the human to be an exception. The basic cell biological processes and interactions discussed here for mitochondria, and emerging principals of cytoplasmic organization such as microzonation and functional compartmentalization, may be central to how developmental competence is established and maintained and, if confirmed for the human, could offer new directions for studies of the impact or regulatory influence on competence of the follicle-specific biochemical milieu. This is an exciting area of research with clear clinical implications, and it is highly likely that as investigations proceed, additional influences of mitochondrial activity and function will be recognized and incorporated into novel protocols or algorithms designed to assess developmental potential and normality.

Review Questions

1. What are mitochondria considered a primary nexus of normal cellular activities that for the human oocyte and nascent embryo are currently considered to be critical determinants of developmental competence?
2. What evidence suggests that the magnitude of the inner mitochondrial transmembrane potential ($\Delta\Psi m$) is important in establishing developmental competence for the meiotically mature oocyte and early human embryo?
3. Why might spatial compartmentalization of differential mitochondrial activities be a necessary occurrence in the oocyte and early embryo with respect to the establishment of normal developmental competence?
4. How are mitochondria in the oocyte and early embryo involved in redox and calcium homeostasis and why might this be developmentally important?
5. What possible functions might bidirection signaling between mitochondria and the nucleus have in the developing oocyte and early embryo, including the human, that could determine normal developmental competence prior to fertilization?

6. Considering current estimates of normal mitochondrial mass and mtDNA content in a human MII oocyte, the multiple roles mitochondria have including a bioenergetic one, and notions of differential spatial compartmentalization, why might the addition of a relatively small number of exogenously derived oocyte mitochondria be expected to shift from developmentally incompetent to developmentally competent, or not?

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Cytoskeletal Architecture of Human Oocytes with a Focus on Centrosomes and their Significant Role in Fertilization

Heide Schatten, Vanesa Y. Rawe, and Qing-Yuan Sun

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Learning Objectives

- Centrosome functions in human oocytes and during fertilization
- Cytoskeletal dynamics during fertilization and development
- Intracytoplasmic sperm injection (ICSI)
- Assays for centrosome functions
- Regulation of sperm aster formation
- Centrosome structure and functions
- The role of specific centrosome proteins in MII oocytes and embryo development

77

77.1 Introduction

In humans and most mammalian species, fertilization takes place at the MII stage (metaphase of second meiosis) in which oocytes are arrested after maturation. Oocyte quality is critically important for successful fertilization, and a variety of different criteria have been used to assess oocyte quality although in many cases the reasons for fertilization failure remain unclear and cannot be determined based on presently available morphological and molecular/biochemical data [1–8, 66]. However, it is important to evaluate specific factors that are known to play a role in oocyte quality and affect successful fertilization. The most prominent structure of the MII oocyte is the MII spindle containing the maternal chromosomes aligned at the metaphase plate and connected to kinetochore microtubules (kMTs) and to acentriolar centrosomes at the opposite spindle poles. Along with nucleation of kMTs from spindle pole centrosomes are pole-to-pole microtubules that are important for chromosome separation (Fig. 77.1). The integrity of the spindle fibers as well as centrosome integrity is an important criterion for oocyte quality as it reflects the general condition of the individual oocyte including oocyte aging that may also occur during the process of IVF [9, 10, 67–74, 76, 77].

Spindle pole centrosomes, whether with or without centrioles, contain numerous centrosome proteins that play a role in specific but also complex centrosome functions as will be detailed in the following sections. The sperm (Fig. 77.2), on the other hand, contains as prominent microtubule cytoskeletal structure the sperm axoneme (sperm tail) and the basal body (a pair of centrioles), one of which (the proximal centriole) will be important for sperm aster formation after fertilization while the other (the distal centriole) is tightly connected to the sperm tail and serves as the nucleation material for the sperm axoneme as will be detailed below. The oocyte and sperm both contribute important cytoskeletal components that are critical for successful fertilization.

The following sections will review the centrosome and microtubule cytoskeletal organization in human oocytes and also in animal models including the pig and bovine models from post-maturation through fertilization and development to first cell division. It should be noted that while the mouse has been used for many genetic studies, it is not a useful model for cytoskeletal organization and fertilization as it differs profoundly in essential aspects compared to all other mammalian species and will not be included in the present chapter.

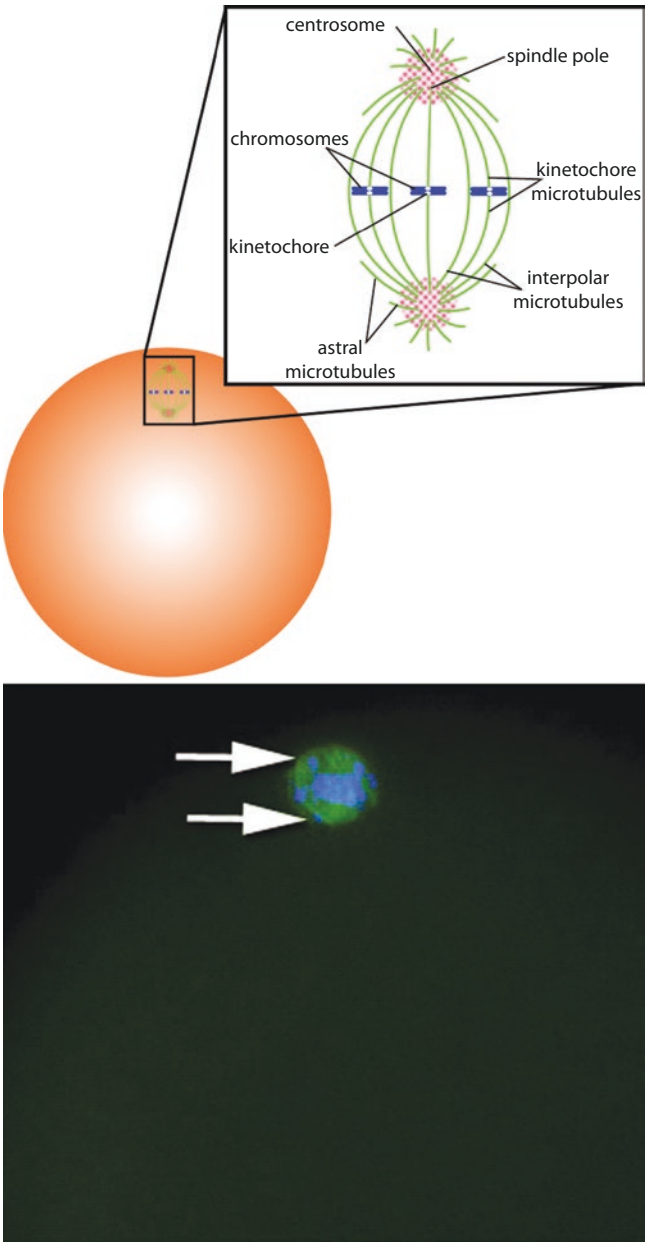

Fig. 77.1 Schematic diagram and immunocytochemistry of MII oocyte before fertilization. The MII spindle is organized from acentriolar centrosomes that nucleate kinetochore and pole-to-pole microtubules that are regulated by a complex set of kinases to hold the MII spindle in shape and prevent deterioration. In most mammalian species, the MII spindle displays perpendicular organization to the egg cortex (parallel orientation in the mouse). Abnormal alignment of chromosomes (blue) can be seen with fluorescence microscopy (arrows, bottom panel). MII spindle microtubules can be detected using antibodies to either alpha or beta tubulin (green)

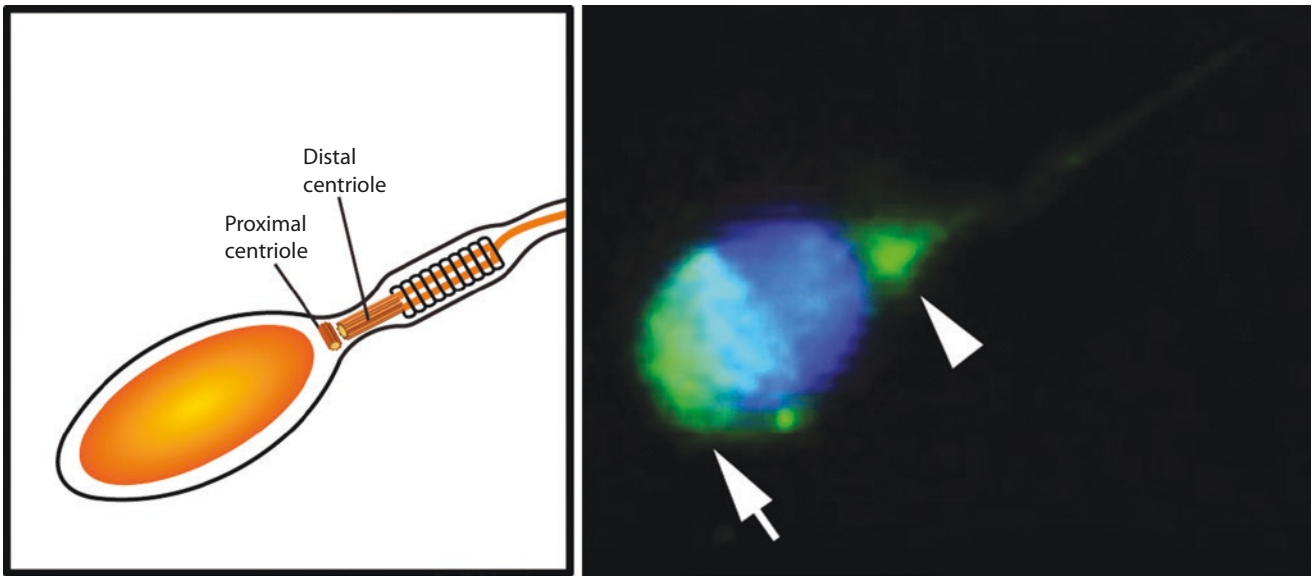

Fig. 77.2 The sperm in most mammalian species contains a proximal and a distal centriole; the proximal centriole is closely associated with the sperm nucleus and will serve as microtubule-organizing center (MTOC) after fertilization, while the distal centriole displays a degenerated microtubule organization and nucleates the axoneme in the sperm tail. A minimal amount of centrosomal material is associated with the sperm centrioles including gamma-tubulin and centrin. Right panel shows ejaculated human spermatozoa labeled with p31 antibody (against proteasome as described in text). Two populations of proteasomes can be observed on the ejaculated spermatozoa: in the acrosomal region (arrow) and in the sperm tail connecting piece (arrowhead). Release of a functional sperm centriole that acts as a zygotic microtubule-organizing center may rely on selective proteasomal proteolysis during sperm penetration, suggesting an important role of sperm proteasomes in zygotic development [11]

77.2 Characteristics of Centrosomes and the Microtubule Cytoskeleton in MII Oocytes, in Spermatozoa, and After Fertilization

The cytoskeleton is mainly composed of three classes of cytoskeletal fibers, i.e., microfilaments (MFs, also called actin filaments or F-actin, 5–9 nm in diameter), microtubules (MTs, 25 nm in diameter), and intermediate filaments (IFs, 10 nm in diameter) as well as numerous cytoskeleton-associated components that play a significant role in cellular functions. However, the centrosome is a critical part of the cytoskeleton and plays a major role in coordinating various cytoskeletal and cellular functions (reviewed in [5, 10, 12–15]. This section reviews the role of centrosomes and the MT cytoskeleton in the MII oocyte and in sperm cells.

77.3 Centrosome Proteins

The structure and composition of centrosomes have been reviewed in several previous papers [5, 10, 12–15] and will only briefly be introduced here. The centrosome is an important microtubule-organizing center (MTOC) that either directly or indirectly is responsible for multiple cellular functions. Lacking a defining membrane that is typical for other cell organelles, the centrosome is a highly dynamic structure that very efficiently communicates signaling functions through its microtubule-organizing capabilities. Numerous centrosome core proteins and centrosome-associated proteins play a role in centrosome functions and direct or control cell cycle-specific events.

A typical somatic cell centrosome is composed of a large number of centrosome proteins surrounding a pair of perpendicularly oriented cylindrical centrioles, therefore referred to as pericentriolar material (PCM). In reproductive cells, the oocyte and the sperm's centrosomal material is reduced during gametogenesis (reviewed by Manandhar et al. [16]), and the mature egg and sperm contain specific centrosome proteins that reconstitute a complete functional centrosome after fertilization. The oocyte does not contain centrioles, but it does contain centrosome proteins, while the sperm contains the centriole surrounded by a small amount of specific centrosome proteins including γ-tubulin and centrin.

The centrosomal core material consists of a fibrous scaffolding lattice whose three-dimensional architecture is primarily maintained through specific protein-protein interactions. It is a highly dynamic structure that compacts and decompacts for cell cycle-specific requirements in which different microtubule patterns are organized depending on the centrosome architecture. Highly compacted centrosomes organize focused microtubule formations, while an expanded centrosome structure organizes various expanded microtubule formations. Gamma-tubulin and the γ-tubulin ring complex (γ-TuRC) are mainly responsible for the nucleation of microtubules, while pericentrin plays a role in recruiting γ-tubulin to the centrosome complex.

Microtubules are anchored with their minus ends to the centrosome core structure ([17]; reviewed in Schatten [5, 10, 12, 74]), and microtubule numbers and lengths are regulated and reorganized throughout the cell cycle; microtubule growth is regulated by distal plus-end addition of tubulin subunits ([18]; reviewed in Schatten [12]; [5, 10, 74]). Microtubules play a role in translocation of vesicles, enzymes, and macromolecular complexes that allow rapid modifications of centrosomal material and impact cell cycle-specific functions in which centrosomal proteins are recruited and dispersed throughout the cell cycle. Rapid microtubule growth and transport along microtubules is especially important for the rapid formation of the sperm aster after fertilization that involves microtubule motor proteins and accessory proteins to reach the female pronucleus for pronuclear movements and apposition and for the union of the pronuclei containing maternal and paternal genomes ([19, 78]; reviewed in [5, 10, 12]).

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77.4 MII Oocytes

The MII oocyte is the end result of a complex process of oocyte maturation that is critical for MII stage-oocyte quality (reviewed in [1, 5, 16, 20–23]). During oogenesis, centrioles that are present in oogonia become disintegrated, and the mature oocyte is devoid of centrioles in most species. However, centrosomal components are present in the MII spindle and in reduced amounts in the cytoplasm (reviewed in Manandhar et al. [16]) that can be visualized in parthenogenetically activated oocytes ([24]; reviewed in Schatten and Sun [13, 14]).

As the oocyte has lost centrioles during gametogenesis, the MII spindle is organized by acentriolar centrosomes consisting of numerous centrosome proteins including the well-known centrosome proteins γ-tubulin, centrin, and the nuclear mitotic apparatus (NuMA) protein. In most species (not in the mouse), the MII spindle is localized perpendicular to the cell surface, and it is a barrel shape to pointed spindle structure (parallel to the egg surface in the mouse which along with many other features re-emphasizes the differences in mouse oocytes compared to most other mammalian species; reviewed in Schatten and Sun [5, 10, 13, 14, 88, 96]).

Although it appears static in immunofluorescence and TEM images, the MII spindle is a highly dynamic structure that maintains its shape by a complex set of regulatory kinases and other regulatory proteins [5, 9, 10, 13, 14, 25, 69, 76, 79–82, 88, 96]. The main functions of the MII spindle are to precisely separate chromosomes and extrude one set of chromosomes into the polar body so that a diploid chromosome set is restored after fertilization during which the sperm contributes the paternal set of chromosomes. Any failure in MII spindle functions can result in cell and developmental abnormalities resulting in abortion, disease, or developmental defects [5, 9, 69, 76, 83]. The MII spindle is therefore an important key structure that requires precise regulation, receiving signals from the surrounding cells, the ooplasm, and the sperm during fertilization. An intact MII spindle as shown in Fig. 77.1 (top panel) is an important criterion to assess oocyte quality. Misaligned chromosomes (arrow in bottom panel of Fig. 77.1) can be visualized as a result of dysfunctional microtubules and molecular motor proteins associated with spindle abnormalities [26].

77.5 Spermatozoa

The components involved in sperm centrosomal functions have previously been reviewed in detail [5, 10, 13–16, 27–30, 88, 96]. Briefly, when spermatids transform into mature sperm, a partial reduction of the sperm centrosome occurs in that the proximal centriole (PC) is retained completely in the sperm with proximal localization to the nucleus, while the distal centriole (DC) becomes partially reduced, and it becomes associated with the sperm axoneme in the midpiece and tail (Fig. 77.2). This distal centriole becomes restructured in that it loses the triplet microtubule organization while a central pair of microtubule doublets becomes apparent, as is characteristic for the axoneme (reviewed in Schatten and Sun [13–15]). Sperm aster organization during human fertilization requires a sperm-derived centriole that must first disengage from the sperm tail connecting piece. Release of the proximal centriole is carried out by sperm (and oocyte) proteasomes during fertilization (right panel Fig. 77.2; [11]).

77.6 The Importance of Centrosomes for Fertilization and Implications for ICSI

As stated above, the precise formation of the sperm aster is critically important for pronuclear movements and for the union of male and female pronuclei as diagrammed in Fig. 77.3. The sperm centriole and surrounding centrosome material is particularly important as it provides the dominant structure onto which oocyte centrosome proteins accumulate to form a functional zygotic centrosome for the formation of the zygote aster. After release of the proximal centriole by proteasomes [11], precise nucleation of microtubules includes precise amounts of γ-tubulin ring complexes (γ-TuRC) composed of γ-tubulin and accessory proteins that are associated with the centrosome core structure and nucleate precise amounts of microtubules in the rapidly changing sperm aster [5, 10, 15, 31, 84–88, 96]. γ-Tubulin needs to be recruited from the oocyte to increase sperm aster size and length in a cell cycle-specific manner that results in the functional zygote aster important for pronuclear apposition. Over-recruitment of γ-tubulin will result in nucleation of too many microtubules, while under-recruitment of γ-tubulin will result in reduced aster formation that both may result in aster formation abnormalities and decreased developmental potential. Studies in the bovine system have revealed that sperm aster formation and

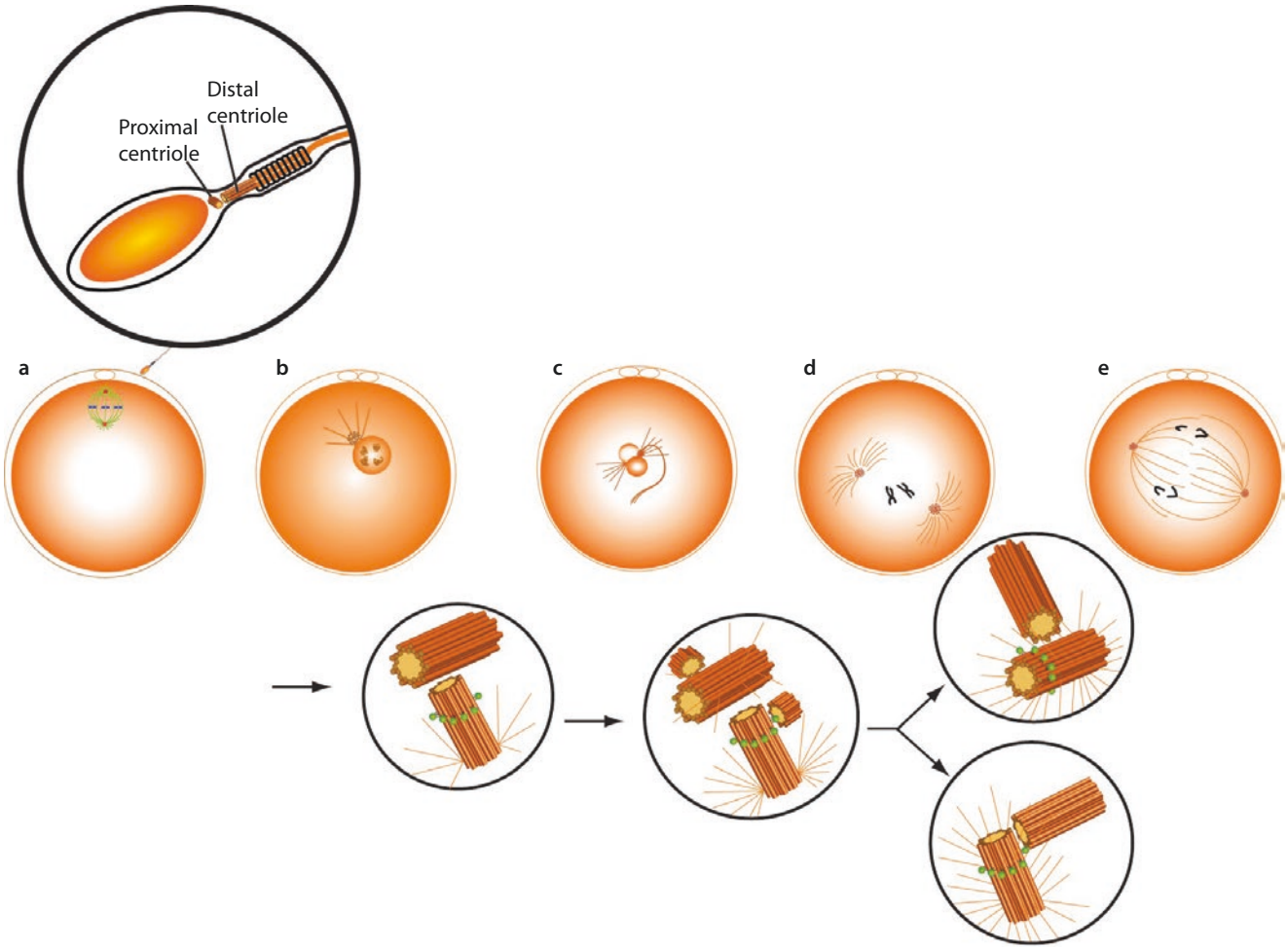

Fig. 77.3 Schematic diagram of the centriole–centrosome complex from fertilization to first cell division. **a** Sperm before fertilization contains a proximal and distal centriole. The meiotic spindle in the MII stage oocyte contains acentriolar centrosomes; **b** sperm aster formation from the sperm's proximal centriole–centrosome complex; **c** after pronuclear apposition, replication of the centriole at pronuclear stage; **d** after syngamy, the duplicated centriole–centrosome complex migrates around the zygote nucleus and relocates to opposite poles to form the centers of the mitotic spindle poles; **e** mitosis of the first cell cycle. Enlarged centriole complex: prior to fertilization, spermatozoa display two distinct centriolar structures with the proximal centriole located within the connecting piece next to the basal plate of the sperm head. This centriole displays a pinwheel structure of nine triplet microtubules surrounded by pericentriolar components. The degenerated distal centriole is organized perpendicular to the proximal centriole and aligned with the axoneme or sperm tail. **b** Shortly after sperm incorporation into the oocyte, a sperm aster is formed from the proximal centriole that allows pronuclear apposition. **c** After pronuclear apposition, the sperm centrioles duplicate during the pronuclear stage (in subsequent cell cycles during the G1/S phases); mother and daughter centrioles form procentrioles, fibrous material that is associated with the proximal region and grows into daughter centrioles (in subsequent cell cycles during the S and G2 phases), resulting in two pairs of centrioles that indicate duplication of centrosomal material. This pattern of centriole duplication is termed semiconservative duplication, as each daughter cell retains one of the mother's centrioles while a new daughter centriole is formed. Centriole and centrosome cycles are tightly coupled, and centrosome duplication occurs at the time centrioles duplicate. **d** The duplicated centrioles separate and migrate around the zygote nucleus to form the opposite poles of the first mitotic spindle. (Modified from Schatten and Sun [15])

size correlated to in vitro embryonic development to the blastocyst stage in which the degree of sperm-derived centrosome and aster organization affected male fertility and early development in a bull-dependent variation [31]. We do not yet know the full range of requirements for optimal sperm and zygote aster formation, but we do know that various factors require precise orchestration that involves phosphoproteins [32] and a variety of other centrosome-associated components that have been studied to a better degree in somatic cells (reviewed in Schatten [12]; Schatten and Sun [5, 10, 13–15, 88, 96]). In somatic cells, perhaps more than 100 different proteins play a role in centrosome and centrosome-directed cell cycle regulations including numerous regulatory components (kinases, phosphatases, and others) that associate with centrosomes during various cell cycle stages. The studies in somatic cells may indicate that over 100 different types of centrosome proteins may be involved in the dynamically changing centrosome composition during aster formation in the fertilized egg. Further studies are needed on basic and molecular levels to determine the full range of requirements for sperm and zygote aster formation to potentially improve in vitro fertilization.

Specific centrosome proteins that we know to play a critical role in centrosome and sperm functions during fertilization include pericentrin and centrin. In somatic cells, pericentrin along with several other proteins plays a role in centrosome and spindle organization [33–35]. Pericentrin forms a complex with γ-tubulin and depends on dynein for assembly onto centrosomes [35]; pericentrin gene mutation results in recruitment loss of several other centrosomal proteins. Centrins are members of a highly conserved subgroup of the EF-hand superfamily of Ca^{2+}-binding proteins. They are important for centriole functions and play an essential role in centrosome duplication ([36–38]; reviewed in [5, 10, 12, 16, 39]). Progress is being made to characterize other centrosome proteins that play a role in centrosome functions during fertilization, but our knowledge on this aspect of fertilization is still very limited.

One other protein that has proven critical for reproduction is the nuclear mitotic apparatus (NuMA) protein that is localized to the MII spindle in the mature oocyte. As NuMA is a nuclear matrix protein during interphase, it is detected in the decondensing sperm nucleus after fertilization [5, 10, 21, 22, 26, 40, 41].

Dispersion of NuMA into the cytoplasm occurs after nuclear envelope breakdown (NEBD), followed by NuMA association with mitotic centrosomes [5, 10, 21, 22, 40]. NuMA is never associated with the interphase centrosome; NuMA localization in the decondensing sperm nucleus after fertilization has clearly been shown for human oocytes [26] and for pig oocytes [40]. Studies in cloned pig and mouse embryos revealed that NuMA contributed by the donor nucleus plays a role in the formation of the mitotic apparatus during first cell division [5, 10, 21, 22, 40–42]. In aging or deteriorating oocytes, NuMA becomes dislocated from the MII spindle. NuMA abnormalities have previously been reported for cloning failures in rhesus monkeys [43].

77.7 Intracytoplasmic Sperm Injection (ICSI) and Assays for Centrosome Functions in ART

Intracytoplasmic sperm injection (ICSI, first reported by [44]) has allowed a novel treatment overcoming male factor infertility primarily related to sperm motility or other unknown factors. The benefits and possible complexities associated with ICSI are important to know (reviewed by Hewitson [45]), as 50% of IVF cycles are now employing ICSI in many IVF clinics (reviewed in [3, 5, 8, 30]). Some of the benefits using ICSI include the possibility to co-inject factors that may be causes of male factor infertility problems which have already been attempted in exploratory studies using the cat as model by co-injecting centrosomal material to restore complete centrosome function (detailed below). Future studies are needed to determine specific factors that are required to restore specific sperm functions after ICSI. Clearly, assessment of centrosomal material in sperm is important. As the important role of centrosomes in ICSI has been recognized, possible therapies have been proposed to restore defective centrosome functions. One of the most frequently used assays to determine sperm centrosomal integrity and functioning comes from indirect studies using heterologous fertilization models in which human sperm and bovine oocytes are used to assess sperm aster formation indicative of centrosome functions [31, 46]. Several heterologous ICSI systems have been employed by various investigators (reviewed in [47, 48]) in which human sperm were microinjected into either rabbit [49, 89, 90] or bovine [19, 50–53] oocytes. These assays established a relationship between infertility and sperm centrosomal dysfunction [54]. Such assays have especially been useful to assess centrosome functions in globozoospermia (characterized by sperm with round heads and lack of an acrosome and acrosomal enzymes and a disorganized midpiece; [55]) in which low rates of sperm aster formation were seen when heterologous ICSI with bovine oocytes was used (15.8%). Understanding the cellular events during mammalian fertilization is a major challenge that is important to pursue for improving future infertility treatments in humans [56, 57]. Figure 77.4 shows several examples of fertilization failures in human zygotes assessed by immunocytochemistry and confocal microscopy. Studies in the domestic cat [58] revealed short or absent sperm asters after ICSI with testicular spermatozoa compared to ejaculated spermatozoa that produced large sperm asters after ICSI. The diminished pattern of aster formation from the testicular sperm centrosome was associated with delays in first cleavage and reduced development to morulae and blastocyst stages, indicating that the size of the sperm aster may predict developmental competence. This aspect is important in light of recent live imaging studies that revealed the critical timing of cell divisions for IVF success rates and embryo development (reviewed in [3, 5, 6, 8]).

The studies in the domestic cat revealed that replacement of testicular sperm centrosome by a centrosome from an ejaculated spermatozoon resulted in higher rates of embryo development comparable to data from ejaculated spermatozoa which indicates that it may be possible to restore centrosome functions with donor centrosomes although ethical questions need to be addressed before proposing such therapies for couples in which infertility is a result of centrosome-related sperm dysfunctions. These studies also point to the possibility that sperm-related centrosome dysfunctions may be associated with incomplete centrosome maturation that is important for centrosome functions and sperm aster formation. Immature sperm centrosomes may play a role in the failure of injection of round spermatids into oocytes (ROSI) that has been unsuccessful when used in IVF procedures [59]. Taken together, these studies reveal a significant role for centrosomes in fertilization indicating developmental potential.

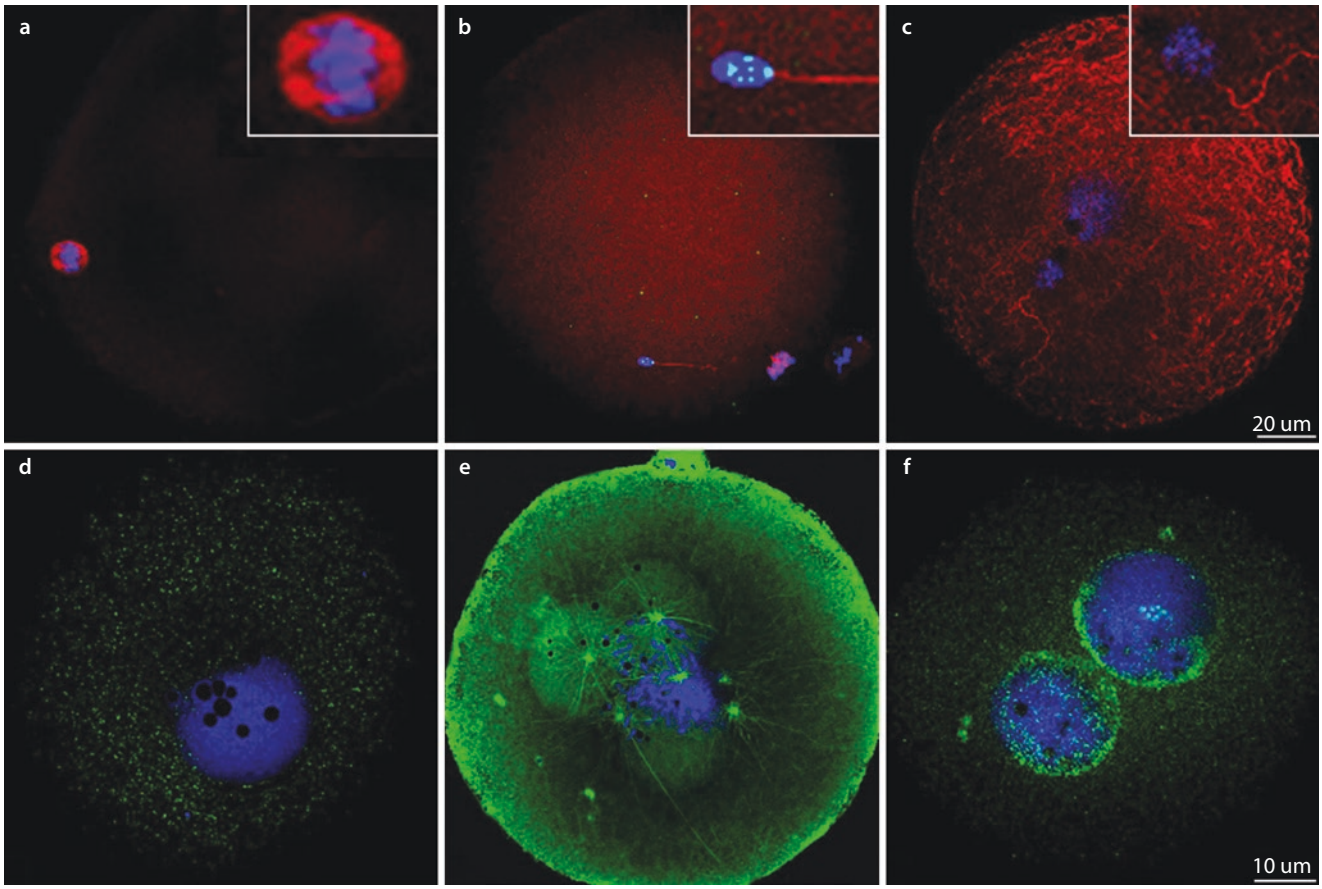

Fig. 77.4 Cytoskeletal organization and DNA configuration after failure and abnormal fertilization during IVF and ICSI. **a** Oocyte meiotic spindle (red: microtubules) with chromosomes (blue: DNA) at the metaphase plate. Inset in the upper right corner displays higher magnification detail. No sperm penetration is visualized. **b** The sperm head failed to form the male pronucleus, and the female chromosomes are still condensed at the meiotic spindle (lack of oocyte activation). Interestingly, fragmented sperm head is visualized after processing sample using TUNEL staining (see detail in the upper right corner, green). **c** Example of failure or incomplete male pronuclear formation at 18 h of ICSI. Microtubules (red) are extended through the entire oocyte cytoplasm as a result of oocyte activation. Male DNA was not completely decondensed (detail at the upper right corner), while female pronucleus underwent decondensation (asynchronous pronuclear development). **d** Formation of one pronucleus following ICSI (blue: DNA), presumably due to the lack of extrusion of the second polar body. **e** Development of three pronuclei after IVF due to polyspermy. Multiple MTOCs are shown in green as detected with anti-tubulin immunofluorescence staining for microtubules. **f** Arrested formation of two pronuclei. Male and female pronuclei are in close apposition at the center of the oocyte's cytoplasm. Nuclear envelopes are seen in green

77.8 Technical Aspects and Practical Considerations

The best characterized centrosomal core protein is γ-tubulin. In *oocytes*, its accurate distribution indicates spindle integrity, as it becomes displaced from the poles when spindles become deteriorated as is the case in aging oocytes (reviewed in Miao et al. [9]).

As indicated above, accurate centrosome function is critical for oocyte maturation, fertilization, and embryo development. As the overall success rate for ART is still low (35–40% in many clinics; up to 60% in others) [91–95], it will be important to study the cell and molecular mechanisms underlying oocyte maturation and fertilization as we still only have an incomplete understanding of cell and molecular criteria to evaluate embryo quality. Our understanding on centrosome formation, composition, and functions in human oocytes is especially poor, and we do not yet have sufficient information on centrosome/cytoskeletal functions and dysfunctions during fertilization and embryo development (reviewed in Schatten and Sun 2011a, b; [5, 10, 88, 96]). We have previously shown that human oocyte maturation, fertilization, and embryo development depend on accurate composition of centrosomes in which NuMA plays a critical role [26]. Previous studies revealed that accurate association of γ-tubulin with the meiotic spindle poles is essential for accurate spindle formation in human oocytes and that dispersion of γ-tubulin from the spindle poles is associated with oocyte aging [97] or with otherwise compromised oocytes that are not competent to be fertilized.

Aging of oocytes can occur during the preparation process which can play a role in human IVF procedures, either in research or in clinical settings. Here, the interpretation of results is particularly critical. As indicated above, during

oocyte aging centrosome integrity is lost in that proteins become dissociated from the centrosome core structure in all non-rodent mammalian species studied so far including humans which results in loss of meiotic spindle integrity. The dispersion of γ-tubulin [97] and NuMA [26] from the centrosome core structure can result in γ-tubulin dispersion and a γ-tubulin association with microtubules [75]. Most prominent and characteristic features in aging non-rodent mammalian (including human) oocytes are numerous small γ-tubulin and NuMA aggregates in the ooplasm. Such cytoplasmic asters are not seen in fresh non-rodent oocytes while, in contrast, they are characteristic for fresh rodent (mouse) oocytes, as rodent oocytes employ different mechanisms for meiotic and early mitotic spindle formation. In the mouse, cytasters are part of the mechanisms employed to form the MII spindle [98, 99, 107] while these cytoplasmic asters that are typical for young mouse oocytes disintegrate during oocyte aging in the mouse [9]. These differences and characteristic features of rodent and non-rodent oocytes are important to know when performing human IVF studies.

The analysis of meiotic spindle integrity is critical for the evaluation of oocyte quality. In recent years, as the demand for IVF and need for IVF success rates have increased, new noninvasive live cell imaging methods have been developed to better assess oocyte quality by imaging and evaluating meiotic spindle integrity as well as other features in maturing and matured oocytes. Several new imaging modalities have been developed to overcome previous limitations (reviewed in [3, 5, 6, 8]). These methods are likely to find applications in IVF clinics to increase IVF success rates.

The still most widely used noninvasive method for the assessment of spindle integrity in IVF clinics is PolScope imaging that has been applied to evaluate human oocyte MII spindle quality [25, 60]. In other test model species including the pig and bovine, immunofluorescence microscopy is typically used to evaluate spindle integrity using anti-tubulin antibodies for microtubules and antibodies to the MII centrosomal proteins γ-tubulin, centrin, and NuMA. Images of an abnormal and normal human oocyte MII spindle are shown in Fig. 77.1 bottom panel and Fig. 77.4a, respectively. It is important to observe that oocyte aging does not occur during the process of in vitro fertilization. In the pig model, several patterns of oocyte aging have been observed which are shown in Fig. 77.5. In humans, such patterns have been observed in deteriorated oocytes, and it has been shown that γ-tubulin and NuMA both dissociate from the spindle poles causing spindle abnormalities [97, 26]. Except for MII spindle evaluation using PolScope optics, future studies may consider live cell imaging with fluorescent centrosome labels such as GFP-centrin which has already been shown effective in pig studies [42].

One other aspect to assess oocyte quality for live cell non-invasive non-harmful imaging is imaging of mitochondrial distribution using multiphoton microscopy that has been employed in hamster oocytes and resulted in successful production of offspring [61]. Such approaches have not yet been used for human oocytes because of ethical considerations, but it may be possible.

Assessment of *sperm* is easier compared to oocytes, as abundant material is available that does not fall under ethical constraints. In sperm, γ-tubulin and centrin are both localized to the basal body and can be analyzed by immunofluorescence or immunoblotting in testing sperm samples. Conventional EM can easily be applied to assess sperm morphology [27, 28], and immunoEM can be applied to determine the accurate localization of γ-tubulin and centrin on ultrastructural levels.

To evaluate *sperm aster functions after fertilization*, still the best indirect assays for centrosome functions available so far are studies of heterologous fertilization in which human sperm is tested for its fertilizing capabilities using bovine eggs as test material. Fertilization can be assessed reliably well in bovine oocytes in which the sperm forms a microtubule-based aster that is indicative of successful fertilization. As mentioned above, the different sizes of sperm asters indicate the sperm's fertilization capabilities and developmental potential. This assay also provides an indirect test for the sperm's centrosomal contributions; its functions can be assessed independent of the human oocyte's centrosomal

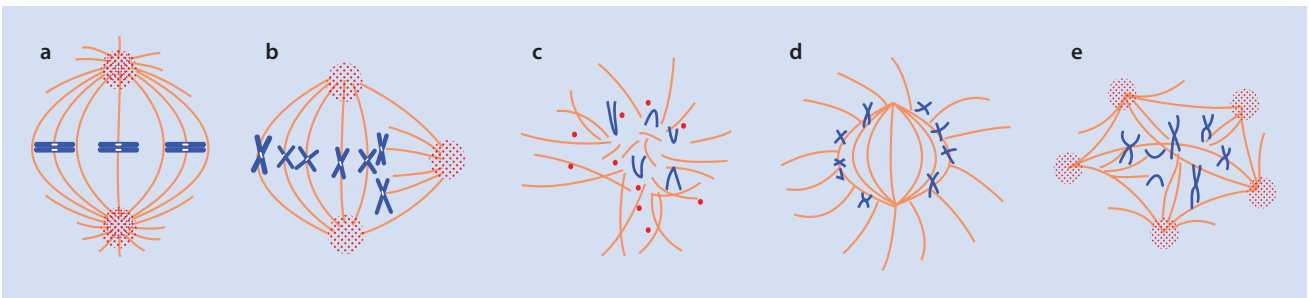

Fig. 77.5 Differences in MII spindle shapes between fresh and aged oocytes. Aged oocytes can display close to normal spindles **a** or highly abnormal spindles **b**–**e**. **b** Tripolar spindle. **c** Highly disorganized spindle with scattered centrosomes and chromosomes. **d** Large irregular spindle in transverse view exhibiting dense staining for tubulin with chromatin attached to the outer edges of the spindle in a rosette formation. **e** Multipolar spindle. Red = centrosomes, green = microtubules, blue = chromosomes. (Modified from Miao et al. [9])

components that may be a contributing factor for infertility, as the oocyte provides γ-tubulin and other regulatory factors that are essential for accurate sperm and zygote aster formation. The heterologous sperm aster tests will allow predictions on sperm or oocyte centrosomal defects. It indicates the sperm's potential to contribute to the fertilization success. If subsequent sperm aster formation is unsuccessful in human oocytes, defects in the oocyte's centrosomal components may need to be considered among other oocyte components that contribute to centrosome and sperm aster regulation. To remedy centrosomal defects, theoretically, various regulatory factors can be co-injected along with ICSI although we currently only know little about factors that play a role in sperm and zygote aster regulation in human oocytes. Experimental manipulation is possible in the heterologous human sperm-bovine oocyte system such as activation by changing pH and calcium factors. While the bovine system has been used for heterologous studies, the pig is increasingly being used as it displays many similarities with human fertilization (reviewed in [5, 10, 13–15, 88, 96]).

As mentioned above, cellular organization of mitochondria has been used by Squirrell et al. [61] to select oocytes for analysis with multiphoton microscopy, a safe method to evaluate live oocytes, and follow development. The observed fertilized oocytes were implanted into a hamster and resulted in subsequent live birth of healthy offspring. Live cell imaging of mitochondria in human oocytes might be worth consideration, as accurate distribution patterns have been associated with positive developmental potential [10, 62, 100, 101]. As mitochondria research in oocytes and developing embryos has progressed significantly [2], new approaches have become available to manipulate mitochondrial functions in oocytes to increase IVF success rates in IVF clinics (reviewed in Ishii [2]; and references therein).

The methods for immunofluorescence microscopy to analyze the microtubule cytoskeleton in human sperm and oocytes have been described in extensive detail by Rawe and Chemes [63]; the following sections in the present chapter are focused on centrosome detection and build on the previous descriptions for microtubules [63] in which media/chemicals, antibodies and staining, consumables and disposables, equipment, and tools have been described in detail. For the centrosome studies of human non-fertilized oocytes and zygotes by electron microscopy (EM), immunocytochemistry (ICC), and fluorescence microscopy in sperm and during ICSI, oocytes and zygotes of cells from couples undergoing ICSI provided the test material for our studies after written consent (described in Rawe and Chemes [63]). Briefly, for the study of human non-fertilized oocytes and zygotes by ICC and fluorescence microscopy during ICSI, cumulus cells are removed followed by removal of the zona pellucida, formaldehyde fixation, permeabilization, antibody labeling, and mounting and visualization of formaldehyde-fixed samples [63]. Examples of applying these methods are shown in Fig. 77.4.

77.8.1 Analysis of Sperm by Electron Microscopy and Immunofluorescence Microscopy

Sperm pathologies comprise a variety of structural and functional abnormalities that are among the many criteria underlying male factor infertility. Abnormally shaped flagella are among the clearly visible pathologies in severely asthenozoospermic men as reviewed by Chemes and Rawe [28] in which dysplasia of the fibrous sheath is most common. Sperm abnormalities can easily be determined with transmission electron microscopy (TEM) of a sperm sample to determine the degree of abnormalities. Centrosomal components can be determined by immunofluorescence microscopy to centrosomal proteins present in sperm before fertilization. Comparison with control sperm samples is recommended. Currently known sperm centrosomal markers are γ-tubulin, MPM2 (phosphoprotein marker), and centrin. Reduced amounts of centrin have been reported in male factor-related fertilization failures [64].

To analyze sperm, a fresh sample of ejaculated sperm is centrifuged and washed in PBS for 5 min as detailed in Rawe and Chemes [63]. After removing supernatant, the pellet is resuspended in PBS. For EM, these samples can now be processed using routine TEM methods as available in electron microscopy facilities.

The procedures for TEM include dilution of sperm in PBS (0.1 M, pH 7.4) (1:4 = sperm/PBS) at room temperature followed by thorough mixing. Next, sperm solution is transferred to a conical tip centrifuge tube and centrifuged at 1500–2000 rpm for 10 min. For fixation, 3% glutaraldehyde in PBS is added at 4 °C for a fixation time of 3–5 h, followed by two rinses in PBS for 30 min each. For second fixation, 1.3% osmium tetroxide (OsO_4) is added at 4 °C for a 2 h incubation time and two subsequent rinses for 30 min each in PBS. Dehydration follows in an ascending series of ethanol at room temperature (50%, 70%, 90%, 95%, 4x100%) for 20 min each followed by three rinses in propylene oxide (20 min each) as transition fluid. Embedding is performed in Epon-Araldite with steps including a mix of 1:1 propylene oxide/Epon-Araldite (2 h), fresh Epon-Araldite changes followed by curing for 24–48 h in EM molds. The materials are available from EM companies with detailed instructions included. Thin sectioning with an ultramicrotome and analysis in a TEM instrument is typically performed in electron microscopy facilities.

Routine TEM methods can be followed by ultrastructural immunocytochemistry to determine the presence of cytoskeletal and centrosome components related to ultrastructure. For the ultrastructural immunocytochemistry procedure, sperm pellets are fixed for 1 h at 4 °C in 5% formaldehyde in PBS (0.1 M, pH 7.4), rinsed in buffer, and dehydrated in an increasing series of ethanol as described for conventional TEM. Infiltration and embedding are performed using LR-White resin, medium grade for polymerization at 60 °C for 24 h. Sections are mounted on 300-mesh nickel grids and

dried at room temperature. Blocking buffer consists of TBS and 10% normal goat serum. Incubation in first antibody is performed by floating grids on a drop of antibody solution and left overnight at 4 °C making sure the samples are not dried out by keeping them in a humidified chamber. Three washes in TBS for 1 h at 4 °C are followed by incubation for 1 h at 4 °C with blocking buffer containing 15 nm colloidal gold-labeled secondary antibody. Sections on grids are then counterstained with 1% osmium tetroxide followed by 1:1 aqueous uranyl acetate/acetone. For negative controls, primary antibodies are either omitted or replaced with primary antibody preadsorbed with excess antigen.

For immunofluorescence microscopy, 100 µl of sperm suspension is placed on a poly-L-lysine-coated coverslip on a slide warmer at 37 °C. After 15 min, sperm are settled and attached to the coverslip which allows easy transfer into fixative (either 2% paraformaldehyde (PFA) in PBS at room temperature pH 7.2–7.3 or 100% methanol chilled to −20 °C) for 40 min. Next, coverslips containing sperm are carefully placed into 6-well dishes (face up) containing 2% PFA in PBS and 1% Triton X-100 to permeabilize sperm cells. Next, for antibody staining, several technical approaches are possible, and one easy approach is to remove the coverslips containing sperm from the six-well plates and place each one on the center cross of a four-well petri dish. A blocking solution is applied for 40 min incubation to bind residual free aldehyde groups. After carefully removing the blotting solution, primary and secondary antibodies are applied as described for oocytes and zygotes. Antibodies for centrosomal proteins include γ-tubulin and centrin.

77.8.2 Analysis of Oocytes by Electron Microscopy and Immunofluorescence Microscopy

The methods for the analysis of oocytes and developing embryos by electron microscopy and immunofluorescence have been described in previous papers and chapters [26, 40–42, 102, 101], and follow the basic methods described above for sperm. In this section, we will only briefly describe the important steps that are different for oocytes and the developing embryo, and we will refer to specific papers in which methods have been described more fully. Excellent methodological details are provided in the methods paper by Rawe and Chemes [63] with step-by-step protocols for sample preparation of human oocytes and embryos. Included are details for materials (media/chemicals, antibodies, consumables and disposables, equipment, tools) and methods (study of human non-fertilized oocytes and zygotes by immunocytochemistry and fluorescence microscopy during ICSI; removal of cumulus cells and zona pellucida, formaldehyde fixation, permeabilization, and antibody labeling; mounting and visualization of formaldehyde-fixed samples, methanol fixation, and antibody labeling; mounting and visualization of methanol-fixed samples). These methods include fixation and processing of single oocyte cells or embryos handled in drops using a mouth pipette, as frequently only few human oocytes and zygotes are available for research. Other methods utilize embedding of oocytes and zygotes in soft agar [102] which is useful when processing for electron microscopy but not for immunofluorescence microscopy. For immunocytochemistry as well as for electron microscopy, all samples need to be completely freed from cumulus cells and the zona pellucida before fixation with formaldehyde or methanol (Zhong et al. [102]; Rawe and Chemes [63], a methods chapter that contains a separate note section for detailed experimental recommendations).

Briefly, for formaldehyde fixation, permeabilization, and antibody labeling, samples are placed in a one-well dish containing 500 µl drops of the respective solutions. Washing between steps is performed by transfer into different drops of washing/blocking solution. When fixing in methanol instead of formaldehyde, 100% methanol is used for 10 min at −20 °C followed by storing coverslips in PBS and 0.1% Triton X-100. Permeabilization is not necessary when using methanol fixation. However, it is important to avoid chilling of samples before fixation when microtubules are being examined, as microtubules are highly sensitive to cold temperature and will depolymerize, resulting in negative results (i.e., absence of microtubule detection).

Antibodies are applied by placing oocytes in 30–50 µl drops of the appropriate dilution of the antibody for 40 min at room temperature or for 1 h at 37 °C under oil. Different temperatures and times are also used by different investigators with no noticeable difference in results. After rinsing and transferring samples into drops of washing/blocking solution, fluorochrome-conjugated secondary antibody is applied in drops for 1 hour at room temperature in the dark, as employed by many investigators, but different times and temperatures are also possible as used by different investigators.

After rinsing again with washing/blocking solution, DNA labeling and visualization follow using confocal microscopy. DNA labeling can employ TOTO-3 at 10 µg/ml for 25 min at room temperature, DAPI or Hoechst 33342 staining at 5 µg/ml Vectashield. For precise details please see methods paper by Rawe and Chemes [63] that contains a detailed notes section to point out potential difficulties and solutions. Here we will address important aspects to consider when performing immunofluorescence staining with centrosomal and anti-α-tubulin antibodies.

The following will provide methods that we have successfully used for detecting microtubules with anti-α-tubulin antibody and centrosomes with anti-γ-tubulin antibody in porcine oocytes [102] which also work for human oocytes.

For *immunofluorescence microscopy of microtubules and centrosomes*, we used antibodies to α-tubulin (1:50; Sigma, Cat # F2168) to stain microtubules and γ-tubulin (1:300; Sigma, Cat# T5192) to detect centrosomes. The zona pellucida of oocytes and embryos was removed using 0.25% pronase, followed by washing in PBS and fixation in 4% (W/V) paraformaldehyde in PBS for 45 min at room temperature.

Three washes in PBS followed before permeabilization in 50% methanol (5 min), 100% methanol (5 min), and 100% acetone (5 min). Extracted oocytes and embryos were rehydrated in 1% BSA PBS for 2 days. Blocking with 3% BSA for 1 h was employed before staining of samples with the primary antibodies, three washes with 1:100 antirabbit IgG second antibodies (F1263, Sigma), and counterstaining with 1 mg ml–1 DAPI in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) to stain DNA. Oocytes/embryos were then mounted on glass slides. All samples were observed with a Zeiss Axiophot, and images were processed with Photoshop software.

For immunofluorescence microscopy it is critically important to perform the appropriate control experiments including the use of primary or secondary antibodies alone to avoid false positives and false negatives. Equally important is the use of unmasking antigens for immunohistochemistry, as epitopes can be masked and not detected by the antibody. Several methods are available to include unmasking of antigens in the immunohistochemistry protocols which are heat-mediated antigen retrieval (also known as heat-induced epitope retrieval or HIER) and enzymatic antigen retrieval.

Enzyme-induced methods are employed using enzymes such as proteinase K (20 g/ml in buffer, pH 8), trypsin (0.5% in dH20), pepsin (0.1% in 10 mM HCl), pronase (0.5% or 0.1% in dH20), and protease (0.5% in dH20) or heat-induced methods which require heat treatment followed by cooling. Chemicals used in this process are citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6), citrate-EDTA buffer (10 mM sodium citrate, 2 mM EDTA, 0.05% Tween 20, pH 6.2), EDTA (1 mM EDTA, 0.05% Tween 20, pH 8), Tris-EDTA (10 mM Tris base, 1 mM EDTA, 0.05% Tween 20, pH 9), and glycine-EDTA (50 mM glycine-HCl, 0.01% EDTA, pH 3.5).

We have employed microwave unmasking (95–98 °C for 10–15 min) to reliably detect NuMA in porcine oocytes [40].

Unmasking of antigen control experiments is especially important when negative results are obtained that may or may not be expected or when different results are obtained by different investigators, as has been the case for γ-tubulin staining in meiotic spindles of human oocytes.

It is also important to point out that different methods can give different results which call for thorough control experiments to eliminate false positives and false negatives. This includes using different fixation methods when immunofluorescence microscopy is performed and includes the above-mentioned antigen recovery methods when negative results are obtained. While most experiments are easy to perform in animal models such as the bovine or porcine system, because of the limited number of available human oocytes, critical control experiments are oftentimes not performed. This may explain the negative results for γ-tubulin that could not be detected at the MI and MII spindle poles under the reported conditions in human oocytes [65] which are different from other studies on human oocytes in which centrosomes were clearly detected as indicated by γ-tubulin staining [97]. Different culture conditions or different preparation methods may explain the different results; future experiments including antigen recovery experiments may provide clarifications on the specific association of γ-tubulin with the acentriolar centrosomes at the meiotic spindle poles of fresh (young, not aging) human MI and MII stage oocytes.

For ultrastructural studies using transmission electron microscopy, oocytes and embryos were fixed for 1 h in 2% glutaraldehyde in 0.1 M PBS buffer, pH 7.4, with 0.05% saponin, and 0.2% tannic acid at various time points. Oocytes and embryos were washed three times and centrifuged at 800 g for 5 min. The supernatant was then gently aspirated while being careful not to disturb the pellet. Then, two percent agar warmed to 50 °C was gently added on top of the pellet and centrifuged to keep the pellet compact. After cooling of the pellet-containing agar, the pellet and a small volume of agar surrounding the pellet were cut out and stored in PBS.

Oocytes and embryos were then rinsed with 0.1 M PBS and postfixed in 1% OsO_4 for 60 min [103–106]. Samples were dehydrated as described above and embedded in Epon resin [105, 106]. Samples were thin-sectioned, followed by uranyl acetate and lead citrate staining before analysis with transmission electron microscopy.

77.9 Conclusions and Future Perspectives

Centrosomes are critically important for sperm and oocyte functions and for successful fertilization after insemination in physiological, IVF, and various ART procedures such as ICSI. Numerous fertilization failures are associated with centrosome dysfunctions and can either relate to sperm centrosomal defects, oocyte centrosome defects, or to regulatory failures after fertilization. Heterologous fertilization models with human sperm and bovine or porcine oocytes will be most useful to more fully analyze sperm and egg centrosomal functions resulting in successful sperm aster and zygote aster formation. Assessing the dysfunctional factors will be important for therapeutic advances. Exploratory studies performed in the cat already revealed that it is possible to restore functional centrosomes in centrosome-related fertilization failures although detailed basic research as well as functional studies are still needed to provide promising new therapies to possibly increase IVF and ICSI procedures related to centrosome dysfunctions.

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Review Questions

1. What is the role of centrosomes in human MII oocytes?
2. What are essential centrosomal and cytoskeletal components contributed by sperm during human fertilization?

3. Why is the mouse an unsuitable model to analyze human sperm quality using heterologous fertilization?

4. When is ICSI used to overcome male factor infertility?

5. Which factors play a role in meiotic spindle aneuploidy?

6. How does oocyte quality affect fertilization?

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Molecular Mining of Follicular Fluid for Reliable Biomarkers of Human Oocyte and Embryo Developmental Competence

Jonathan Van Blerkom

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Learning Objectives

- Why follicular fluid is considered a viable source of biomarkers for oocyte and preimplantation embryo developmental competence in clinical IVF?
- The types of molecules in follicular fluid that have shown promise as competence biomarkers.
- Which analytical methods for biomarker detection and quantitation may be the most practical for routine IVF programs to introduce for competence assessment.
- Problems associated with clearly establishing a physiologically significant and predictive relationship between follicular fluid components and developmental competence.

Current advances in the development and application in clinical IVF of microanalytical imaging, genetic, and biochemical technologies lead to the interesting possibility that in the near future, competence assessments of human oocytes and embryos will be algorithm-based rather than operator-based. In such a future, selection for insemination and single embryo transfer would rely on a combination of findings assessed at selected phases of oocyte and early embryonic development. For the embryo, quantifiable biometric characteristics of developmental performance in vitro obtained by time-lapse imaging would include pronuclear morphology and nucleolar organization, the timing and uniformity of cleavage divisions, the presence of micro- and multinucleation in blastomeres, and an accurate value for the degree of cytoplasm lost to fragmentation, if any [1]. Biochemical criteria for competence selection could come from a variety of current methods such as microanalysis of spent culture medium to detect molecular signatures [2–4], levels of gene expression by cumulus cell [5, 6], and genetic status determined by high-resolution genomic analyses from blastomere and trophectoderm biopsies [7]. In this future, the human factor is necessarily relegated to ovum pickup, perhaps fertilization, and most likely embryo transfer.

Whether this scenario is a realistic one remains to be seen, as earlier and sanguine predictions of biochemical or physiological biomarkers of oocyte competence have yet to be fulfilled [8, 9]. However, the need for competence assessment, whether driven by instrumentation or observer, arises from an undeniable biological fact; namely, that with respect to outcome, each human oocyte and each human embryo have a unique developmental potential and that often, competence is already compromised in the mature and fertilizable oocyte [10]. This notion has been repeatedly validated by millions of IVF cycles performed over the past three decades. Although not unexpected, the results of the chromosomal assessments of meiotically mature (metaphase II, MII) human oocytes and early cleavage-stage embryos with different high-resolution methods have consistently demonstrated high frequencies of aneuploidy, often with multiple chromosomal anomalies (trisomies and monosomies) affecting the same oocyte [11], and for the early embryo, aneuploidy and other developmentally lethal chromosomal segregation disorders (e.g., mosaicism [12, 13]). The occurrence of these defects still comes as a surprise to even the most experienced observer when morphologically normal and stage-appropriate oocytes and preimplantation-stage embryos are found to be chromosomally abnormal.

If computerized biometric analyses of oocytes were to become standard methods to select oocytes for insemination and embryos for transfer, the underlying causes of differential competence, such as aneuploidy, would need to be detectable in observer-free systems. It is doubtful that morphological characteristics that may be associated with competence (e.g., polar body volume, zona birefringence, cellular debris in perivitelline space [1]) can identify aneuploidies and other chromosomal defects either in the oocyte or in the early embryo. Likewise, there is no compelling clinical or experimental evidence to date which suggests that early embryos carrying specific chromosomal or single gene defects produce unique molecular signatures detectable in spent culture medium or, for that matter, can be identified by the over- or underexpression of specific mRNAs or proteins in a biopsied blastomere. This may change as human embryos with known chromosomal and genetic defects are subjected to highly sensitive analytical methods that can characterize biochemistry in microliter volumes or the molecular biology of a single cell (see below).

78.1 Follicular Fluid Biomarkers of Oocyte Developmental Competence

The efficacy and sensitivity for competence selection at the human oocyte and early embryo stages that may be afforded by molecular and metabolomic analyses remain to be demonstrated. In this regard, their use for purposes of selection may be problematic as both activities represent dynamic processes that can be subject to the conditions of culture. As discussed in detail by Menezo and Guerin [14], levels of gene expression, metabolism, and other bioactivities are not only oocyte and embryo specific but can also be significantly influenced by culture medium composition (e.g., amino acids) and the conditions of culture (e.g., oxygen tension). It can be concluded from this report that because of the dynamic nature of the interaction between the environment and the cellular processes that produces potential competence biomarkers, each needs to be evaluated critically in general and for embryos in particular, as early development from fertilization through the preimplantation stages is entirely in vitro. In addition, cost vs. benefit calculations need to be taken into account based on unambiguous demonstrations of improved outcome, as well as the ease with which genetic microarray, metabolomic, proteomic, and time-lapse imaging methodologies can be incorporated into the routine clinical IVF laboratory.

It has long been thought that competence biomarkers should exist in the follicular fluid given the differences in maturational state, fertilizability, and development competence observed in oocytes aspirated from follicles that show equivalent patterns of growth and development under

exogenous gonadotropin stimulation (controlled ovarian hyperstimulation, COHS). The underlying issue in the search for competence markers in follicular fluid is related to the relative contribution of external influences that can be demonstrated to be developmental regulators or drivers for the oocyte vs. intrinsic differences that occur in oocytes themselves (i.e., at the molecular, cellular, and chromosomal levels). In this case, adverse downstream developmental consequences that may occur after fertilization would be expected to be independent of the biochemical environment within the follicle.

The search for external influences on competence has logically focused on the intrafollicular environment to which the oocyte is exposed. However, the fluid obtained for analysis may not represent conditions that could influence competence during the relatively prolonged FSH-dominated phase of follicular growth and development, but rather what occurs some 36 h after ovulation induction, typically with HCG rather than LH. Further complicating this type of analysis is that the vast majority of IVF cycles are hyperstimulated, and comparisons to natural (spontaneous) are virtually nonexistent, and COHS treatment could distort relative concentrations of regulatory factors, especially when fluids are pooled rather than analyzed individually for each follicle.

With these caveats in mind, what has made the follicular fluid so appealing for investigations of regulatory influences on human oocyte developmental potential is that the biochemistry is both dynamic and extraordinarily complex, a mixture of steroid hormones, growth factors, gonadotropins, cytokines, ions, amino acids, lipids, reactive oxygen species, enzymes, and other bioactive molecules that are produced in situ from the mural and cumulus granulosa or which pass through the blood follicle barrier as transudates from the perifollicular capillary bed [15–33]. It is clear even from this relatively short list of reports that qualitative and quantitative analyses of human follicular fluid demonstrate no shortage of molecules that could be biomarkers of competence, including those with regulatory and signaling functions. One of the foremost challenges in undertaking this type of investigation is the necessity to distinguish between potential targets for investigation that (a) actually influence or regulate oocyte processes and lead to competence, (b) those whose bioactivity is specific to the somatic cells of the follicle, or (c) are secretory products of the cumulus and mural granulosa destined to enter systemic circulation and whose activity is extrafollicular. However, since the beginning of clinical IVF in 1978, and despite a relatively enormous literature on this subject, few have been shown to have meaningful predictive value for selection, and at present, follicular fluid is not used for analytical purposes and is discarded by most programs. Another basic question that is rarely addressed in reports of the utility of follicular fluid for competence assessment is whether a potential biomarker can be shown to function through a signaling cascade that operates in the oocyte. In this regard, the presence of a particular growth or regulatory factor cannot be assumed, a priori, to be targeted to the oocyte, however appealing such a notion may be to those involved in competence studies. Perhaps, this is why so many reports of suggested biomarkers have been contradictory with respect to competence and outcome.

Further complicating the identification and characterization of bioactive molecules and regulatory factors that may be clinically useful as competence biomarkers in clinical IVF is whether they directly influence the developmental biology of the human oocyte or the cumulus and coronal cells. The presence of an intervening acellular zona pellucida is both a physical barrier between the oocyte and the follicular milieu and a biological filter that limits the diffusion of molecules to those up to ~60,000–70,000 daltons. Here, the relevant issue is the particular manner by which they enter the perivitelline space (the immediate environment of the oocyte) or pass into the cytoplasm directly from the corona radiata and proximal cumulus oophorus (the somatic cell compartment) by means of gap junctions between the oolemma and the transzonal processes (TZPs, see below).

The likely pathway for the transmission of potential regulatory molecules is by means of TZPs, which arise early in oogenesis (coincident with zona pellucida formation) and occur as dense circumferential network of slender extensions of the corona radiata, the cells that reside on the zona pellucida and, to a lesser extent, from cells of the cumulus oophorus in proximity to the corona radiata [34]. These processes permit bidirectional communication between the developing and fully grown oocyte and its somatic cell component by means of gap junctions formed by connexin 37 hexamers. Transmission electron microscopic images of the TZPs show longitudinal arrays of microfilaments that extend from the cell body to the site of contact with the oolemma, and it has been suggested that they provide an internal architecture to facilitate directional transport [35]. More recently, the presence of mitochondria in TZPs has been demonstrated in living cumulus-oocyte complexes stained with mitochondria-specific fluorescent probes [36] with elongated, high-potential organelles detected along the entire length of the TZPs. Mitochondria located in proximity to the TZP terminus on the oolemma could supply ATP directly to the oocyte through gap junctions and supplement the endogenous bioenergetic capacity of the GV-stage ooplasm in the subplasmalemmal and pericortical cytoplasm, where the demand for ATP may be higher than in more interior regions [36].

While intercellular communication by the TZP pathway persists up to the luteinizing hormone (LH)-induced resumption of arrested meiosis, gap junctions are primarily involved in metabolic and electrical coupling between cells and function in this regard by regulating the flow of small molecules that act as secondary messengers. These junctions generally restrict passage of molecules to those approximate 1000 daltons, such as cyclic AMP, ATP, ions (e.g., calcium), and small polypeptides, but not proteins the size of most growth factors, including gonadotropins. Therefore, the potential developmental influences of regulatory proteins and other factors suggested to affect oocyte competence that exceed the molecular weight limitation of gap junctions are unlikely to do so

by this direct pathway of intercellular communication. So what does the oocyte actually "see" at its surface and how do extrinsic regulatory signals or developmental cues arrive in the perivitelline space, given the notion of the zona pellucida as a selective molecular filter, if not a barrier to certain macromolecules? For proteins excluded by gap junctions to affect the oocyte, receptor-mediated signal transduction at the level of the oolemma and uptake by endocytosis are the obvious means.

The central issue here that is worth repeating is that investigations of follicular fluid designed to identify molecules that may be involved in the acquisition of developmental competence need to consider the mechanism by which they can directly or indirectly affect the biology of the oocyte. For example, despite the withdrawal of TZPs from the oolemma at the outset of resumed meiotic maturation, the processes and the cells from which they originate remain intact and functional, as indicated for the latter by the presence of mitochondria that retain high potential [36]. Scanning electron microscopy of the underside of the zona pellucida in maturing and mature human oocytes shows a dense circumferential network of residual processes in the perivitelline space that in the native state remains in close proximity to the oolemma [34]. Therefore, synthetic and secretory activity by the corona radiata and proximal cumulus granulosa likely continues during preovulatory maturation, and putative influences from the follicular fluid on cumulus and coronal cells that could influence the oocyte likely persist. However, it remains to be determined whether biosynthetic activities that could affect the human oocyte change qualitatively or quantitatively in vivo under the influence of LH during the ~36-h-long preovulatory period. If such changes are confirmed, the long-held notion that termination of TZP-mediated communication at the GV-stage signals a fundamental shift from maternal to oocyte regulation of development may need to be reconsidered.

The persistence of information flow between residual somatic cells and the oocyte during preovulatory maturation may be a currently unrecognized aspect of how competence is established, especially if the molecular nature of this information flow changes as maturation progresses to ovulation. At present, there is no evidence to suggest that as long as granulosa cells continue to secrete proteins into the perivitelline space during the preovulatory period, uptake by the oocyte (receptor mediated or endocytotic) is not functional during its maturation [37].

The intent of the preceding discussion was to emphasize that the presence alone of well-characterized growth factors and other regulatory molecules in human follicular fluid is insufficient to assume that the oocyte is the target or that they influence developmental competence. It may be for these reasons that despite the complex array of potential regulatory factors in follicular fluid noted above, to date, unambiguous evidence for developmentally significant effects on oocyte competence has remained elusive. It may well be that the principal target for growth factors in follicular fluid is the granulosa compartment, first by upregulating cell proliferation and steroidogenesis by mural granulosa and subsequently, to prepare both mural and residual cumulus granulosa for the transition to a vascular corpus luteum [38]. This is likely the function of angiogenic factors such as VEGF [39] and leptin [16], which occur at relatively high concentrations in preovulatory human follicular fluid.

While the follicular fluid can be a "gold mine" of potential regulatory factors, the fluid is discarded by virtually all IVF programs. Further complicating any scheme of molecular analysis is the unavoidable fact that follicular aspiration is not a "clean" process, and for analytical purposes, each follicle must be aspirated individually and with rinses of the aspiration needle between punctures to prevent cross-contamination with residual fluid and blood. The collection of neat aspirates can be time-consuming and can significantly extend the length of the ovum pickup procedure, especially when numerous follicles require puncture. The collection of individual aspirates also entails the tracking of the corresponding oocyte from fertilization through transfer, which, while feasible, adds considerable time and effort to the laboratory routine. Therefore, the selection of potential biomarkers of competence requires some degree of confidence that based on the known bioactivities of a candidate molecules; there is a high probability that its function, either through the cumulus and coronal cells or on the oocyte directly, will be developmentally significant.

With the possible exception of Mullerian-inhibiting hormone (see below), no single component of the follicular fluid has met these criteria to date, and reports that some might, such as VEGF and leptin, have not been proven or are controversial [40–43]. Likewise, it remains to be determined whether mRNA profiles of cumulus cells, collected either free floating in aspirated follicular fluid or mechanically detached from the oocyte in vitro, are sufficiently predictive of outcome as to warrant the additional infrastructure required for microarray analysis and, more importantly, meaningful interpretation. Comparisons of mRNA expression profiles that can show differences between individual oocyte-cumulus complexes from the same or different ovaries offer a promising approach to competence selection, but whether it is clinically applicable will only become apparent when a core set of genes with known functions is identified and, on the basis of outcome, demonstrated to consistently distinguish between mature oocytes.

Recent studies of Mullerian-inhibiting hormone (AMH) concentrations in follicular fluid and outcome results after IVF suggest that this molecule may indeed be a competence biomarker. AMH is a dimeric glycoprotein member of the transforming growth factor (TGF)-β superfamily whose expression by granulosa cells in large preantral and small antral follicles is upregulated at the transcriptional and post-transcriptional levels. Serum levels of AMH have received considerable attention as an indicator of ovarian reserve and for predicting the unique response of women to controlled ovarian stimulation, as well as biomarker of oocyte developmental competence [44, 45]. There is accumulating evidence that follicle-specific levels measured in aspirates at ovum

retrieval may indeed be related to outcome after transfer (reviewed by Van Blerkom and Trout [9]). Based on IVF outcomes, Eldar-Geva et al. [46] proposed that of all the factors identified in follicular fluid up to that date, only AMH appeared to be a reliable biomarker of developmental competence for the oocyte and resulting embryo.

While the collection and preparation of follicular aspirates impose special requirements to assure the validity of AMH quantitation, the availability of commercial ELISA-based assays permits rapid results that can be used as an independent variable in oocyte and embryo selection schemes that include such traditional parameters as stage-appropriate development, performance, and morphology during in vitro culture. However, despite the growing evidence of the value of follicular AMH determinations, confirmation of optimistic reports that this protein hormone can be a highly meaningful predictor of outcome will become evident only after more IVF programs combine follicle-specific AMH concentration with outcome results from the corresponding oocyte. It may take some time to confirm or reject AMH in this regard, as most IVF centers adopt a new protocol only after sufficient confirmation is forthcoming, which is typically not from their own independent studies but from the often laborious efforts of a very few investigators.

AMH is an attractive candidate as a biomarker of competence because it is likely that levels within a specific range reflect the normality of granulosa cell development and function, which, in turn, would be expected to influence the normality of the oocyte as the follicle develops and enters the preovulatory pathway. As a member of a signaling cascade (TGF-β superfamily) that has been extensively investigated in multiple species, AMH represents an ideal candidate for detailed gene expression and function studies designed to characterize precisely how it may regulate or influence the acquisition of human oocyte competence.

78.2 A *Holistic* Approach to Follicular Fluid in Competence Selection

Analytical methods that offer a detailed molecular profile of neat follicular fluid have the real potential to provide a detailed biochemical "picture" of the intrafollicular milieu that may ultimately be of greater clinical utility as a biomarker of competence than are levels of individual molecules, including AMH. Methodologies such as mass spectroscopy, nuclear magnetic resonance (NMR), and Raman spectroscopy (near-infrared spectroscopy, NIR) can display molecular profiles or signatures of a wide array of molecules (e.g., amino acids, metabolite small bioactive peptides, and proteins) whose levels can be correlated with embryo performance in vitro and outcome after transfer [47–50]. Similar to the rationale upon which metabolomic analysis of spent culture medium has been proposed for purposes of preimplantation-stage human embryo selection [2, 4], this "holistic" approach to follicular fluid analysis looks at both the end products of cellular activities and the presence of molecules that enter the follicle during its growth. Because instrumentation to perform NMR and NIR analysis can be adapted for use in the clinical IVF laboratory, it is likely that yet another algorithm will ultimately replace the observer for both oocyte and embryo selection, assuming that predictability levels are ultimately found to be robust. This should not be considered a negative in clinical IVF because current empirical assessments of competence based on cumulus characteristics (size, degree of expansion, the presence of foci of red blood cells, cytoplasmic density) are all that can be effectively noted, but their relevance with respect to outcome is unclear, controversial, and in some instances, more apparent than real [1]. While the human element becomes the means by which an analytical end is achieved, the potential for establishing standard, objective criteria for selection based on molecular profiles derived from the high-resolution methods noted above should be welcomed in clinical IVF laboratory. However, only the continued accumulation of outcome-based findings will demonstrate whether the current optimism that they can indeed be robust and reliable predictors of competence is justified.

78.3 Perifollicular Blood Flow as a Noninvasive Predictor of Oocyte Competence

Interest in Doppler ultrasonographic analysis of perifollicular blood flow rates to assess the normality of follicular growth and oocyte competence has been episodic since it was first introduced in clinical IVF in the late 1980s and early 1990s (see reviews by Gregory [51]; Van Blerkom and Trout [9]; Van Blerkom [52]). While most of the early reports were generally positive with respect to oocyte and embryo selection, few clinical IVF programs incorporated Doppler analysis in their follicular monitoring schemes, even when the capacity to obtain spectral imaging and quantitative values of follicle-specific blood flow was available in their instrumentation. Renewed interest in this noninvasive method of follicular analysis may be attributed in part to two factors: (a) the need for oocyte selection criteria that are independent of cumulus morphology at aspiration, especially where the number of oocytes that can be inseminated (or embryos transferred) is mandated by law and (b) the introduction of new generations of ultrasound equipment that produce high-resolution, 3D digital images that can be manipulated in real time. The ability to digitally isolate individual follicles and view blood flow patterns along the entire circumference of the follicle wall may be an important diagnostic tool and is in contrast to older 2D imaging modes, in which blood flow images and quantitative parameters (e.g., resistivity index) were obtained from selected cross-sections.

The physiological basis for assuming that perifollicular blood flow measurements offer some insight into the normality of follicular development and the competence of the corresponding oocyte is that expansion of the existing

perifollicular vascular bed is normal aspect of folliculogenesis in follicles in the ovulatory pathway. Expansion of the microvasculature network appears to involve specific angiogenic growth factors such as VEGF and leptin (see above) produced by cumulus granulosa cells under the influence of FSH and LH [53]. VEGF also increases the permeability of capillaries (it was originally termed vascular endothelium and permeability factor) that might enhance the transduction of blood-borne regulatory factors into the follicle. Higher rates of blood flow would also increase rates of oxygen diffusion into the follicle, as well as the rate at which follicular components (steroids, growth factors, etc.) enter systemic circulation. Increased follicular oxygenation may be an important regulatory influence for the steroidogenic activity of the mural granulosa cells that line the follicular wall and are in close proximity to the perifollicular microvasculature. Despite numerous studies of follicular vascularity and steroidogenesis, it remains to be determined whether the level of estradiol measured in serum during follicular growth or of progesterone after ovulation induction can be related to follicular blood flow characteristics in general or whether high-flow follicles contribute disproportionately to levels measured in serum.

Differences in blood flow rates detected by Doppler imaging have been positively correlated with corresponding differences in the dissolved oxygen content of follicular fluid measured in neat aspirates obtained after ovulation induction in COHS cycles for IVF [54]. Although the reported differences are relatively small (i.e., between ~1 and ~4%), they may be physiologically significant insofar as reducing the extent of hypoxia that normally exists within the follicle [53] which, in turn, could influence the bioactivity of both mural and cumulus granulosa cells [54]. Molecular studies indicate that follicle-specific levels of VEGF in follicular fluid appeared to be related to corresponding expression levels of elements of the hypoxia-inducible transcription factor-signaling pathway (HIF) [9, 53], which regulates levels of VEGF expression [55]. It has been suggested that the activation of HIF may be associated with FSH stimulation of granulosa cell expansion and that the level of dissolved oxygen within the early antral follicle could be rate limiting for both granulosa cell proliferation and steroidogenic function [53]. Although speculative, one indirect action of FSH on granulosa cells could be at level of the mitochondria, which, as the oxygen sensors of a cell, could respond by increasing superoxide production to levels that are regulatory with respect to the activation of the HIF pathway [56]. Collectively, progressive increases in dissolved oxygen content during the early follicular phase may regulate granulosa cell proliferation, levels of steroid production by the mural granulosa, and protein growth factor synthesis and secretion by the cumulus granulosa. This notion is supported by the findings of Shrestha et al. [57], who distinguished between "good" and "poor" beginners on the basis of perifollicular blood flow rates measured during the early stages of follicular growth in cycles of controlled ovarian stimulation for IVF. Based on outcomes after embryo transfer, they concluded that flow rate, implantation potential, and developmental competence were related to such an extent that a poor beginning could justify cycle treatment cancelation during the early stages of stimulation.

It is worth noting that while increased intrafollicular oxygen tension levels seem relatively small (<1% to approximately 4%) [54], they may be of a magnitude sufficient to influence the function and activity (e.g., gene expression levels) of the mural and cumulus granulosa cells during follicular growth. For the cumulus granulosa in particular, levels of biosynthetic activity during the follicular phase could indirectly influence the normality cytoplasmic, nuclear, and oolemmal maturation during preovulatory period. In this regard, significantly lower frequencies of aneuploidy at MII have been reported when the dissolved oxygen content measured at aspiration was approximately 4%, as compared to similar-sized follicles with poor flow characteristics and an oxygen contents ≤~1% [54].

One of the more unexpected findings to come from the early studies of perifollicular blood flow was the extent to which follicles of equivalent size at the time of aspiration, including those adjacent follicles, exhibited completely different quantitative flow values and in some reports, high-flow follicles occurred in one ovary, in a single follicle on one ovary, or in multiple follicles on one or both ovaries. Thus, blood flow rate could not be predicted on the basis of follicle size or location without Doppler analysis [54]. In order to quantify perifollicular blood flow rates, relatively simple grading systems were proposed using a score (A, B, C; 1–4) or grade (high or low grade) that was based on degree to which flow could be measured, either in a midline section or at multiple points, along the circumference of the follicle [51]. Correlations between blood flow characteristics, fertilization, embryo performance in vitro, and outcome indicated that oocytes from high (type A; class 3 or 4) grade follicles were more likely to result in pregnancy than those from low-flow (grade) follicles (see reviews by Gregory [51]; Van Blerkom and Trout [9]). However, while usually positive correlations between outcome and perifollicular blood flow rates appeared in the literature (see above), indicating that this metric could be used as an independent factor for oocyte and embryo selection [58], Doppler ultrasonographic analysis of perifollicular blood flow characteristics has not been widely incorporated in infertility assessment and treatment. In the past, the apparent lack of interest in this methodology may be due to the requirements for instrumentation that could perform Doppler studies, the added time, and expertise needed to obtain accurate values with conventional 2D imaging or that the association with outcome was not sufficiently high as to warrant a significant change in protocol.

Renewed interest in blood flow measurements largely parallels the introduction of digital 3D ultrasonographic imaging in which Doppler software is often included with the instrument. Many, but not all studies, have confirmed an association with outcome and for some significant reductions in spontaneous miscarriages, supporting earlier findings that

aneuploidy may be less likely in oocytes that mature in high-grade follicles (reviewed by Van Blerkom and Trout [9]; Van Blerkom [52]). While not all reports have been sanguine with respect to the utility of Doppler imaging in IVF treatments, reports of improved outcomes, including higher ongoing pregnancy rates and reduced frequencies of miscarriage, do suggest that its inclusion in ovarian monitoring and oocyte and embryo selection schemes is beneficial and can provide a quantitative measure of follicular development that is independent of growth rate. What 3D imaging has shown however is that in comparison to single cross-sectional 2D views, perifollicular blood flow in high-grade follicles cannot be assumed to involve the entire circumference of the follicle [9]. In these instances, high flow can be focal and discontinuous with relatively large regions of the follicular wall showing little, if any, significant velocity. While these follicles would likely be classified as high grade by 2D Doppler ultrasonography, they are more likely moderate to low grade; whether the corresponding oocytes have a lower competence with respect to implantation and outcome than their counterparts from follicles where blood flow is largely continuous and circumferential remains to be determined. Therefore, the simple follicular classification schemes noted above might need to be revised and standardized in order to account for subtle differences in perifollicular blood flow in apparently high-grade follicles that may be significant with respect to competence selection.

Perhaps, the most convincing evidence for the use of Doppler analysis of follicles will come from the type of NMR and NIR profiles of follicular fluid noted above, assuming that such studies will be able to show molecular signatures and levels that can produce algorithms that clearly correlate with outcome. In the meantime, where this technology exists, its use for follicular assessment should be considered as the first step in sequential assessments of competence that after fertilization include the usual morphological characteristics of pronuclear through blastocyst-stage embryos [1].

The notion that subjective observations of early human development commonly used to assess embryo viability will be succeeded by objective criteria that can be expressed in a numerical form that is predictive of outcome, such as proposed for NIR values obtained from spent embryo culture medium, is an appealing one because it would be derived from quantitative measurements of follicular characteristics (e.g., blood flow rate and pattern assessed by 3D Doppler imaging) and biochemical profiles of neat follicular fluid [59]. A change of this type in how the clinical IVF laboratory is engaged in competence assessments should be viewed positively and in terms of the potential to improve outcome and, more importantly, equalize outcomes among programs.

78.4 Summary and Perspectives

The search for biomarkers of oocyte and embryo developmental competence has been ongoing since IVF was combined with COHS to become a practical and widespread treatment for human infertility. The early optimism that measures follicle-specific steroid hormone, protein growth factor, cytokine, and other bioactive molecules detected in the complex biochemical mix that is the follicular fluid could be biomarkers of gamete, and embryo competence has not been supported by a large body of research. Thus, the biochemical and physiological environment to which the cumulus-oocyte complex is exposed to prior to ovulation is represented by the fluid discarded by most clinical IVF laboratories. Whether the current enthusiasm for some factors, such as AMH, may prove to be the exception remains to be determined. In the same respect, whether molecular surveys of gene expression in cumulus cells, either at the mRNA or at the protein levels, have sufficient predictive power to warrant adoption as a routine protocol for assessment remains to be seen. While there is no shortage of potential targets for analysis, and studies to screen targets and identify a core set of proteins or genes that may be related to competence are ongoing in this field, it is unclear at present whether they will have sufficient predictive power for oocyte and embryo selection to justify the considerable increase in expense (e.g., custom microarrays, equipment, and technical expertise) and effort (e.g., biopsy and preparation of cumulus cells) that may be required.

The "holistic" or spectrophotometric approach to competence assessment with NMR, NIR, or similar analytical methodologies is intended to obtain a comprehensive molecular snapshot of the intrafollicular milieu at the time the oocyte is retrieved. In this instance, it is the "big picture" that is relevant rather than whether the function of a putative biomarker is on the somatic cells or female gamete or both. The appeal of this line of investigation is twofold: first, the results can be both qualitative and quantitative, and second, the molecular profile displayed should be consistent with the intrafollicular biochemistry in which the oocyte matured to MII and achieved fertilization competence. In this respect, its utility lies in the fact that the analysis is done at a critical developmental endpoint for the oocyte, the transition from intrafollicular life, where the components in the follicular fluid are those derived from serum or produced in situ, to a different biochemical milieu within the Fallopian tube, where fertilization will occur.

The importance of obtaining quantitative values is that if specific components are shown to be competence associated, it may well be that it is their concentration rather than simply their presence that is associated with the acquisition of developmental viability. What will determine the success of this approach is whether comparative analysis of individual follicles reveals a relatively small number of biomarkers that can reliably distinguish oocytes that develop into embryos that progress from gestation to birth from those that do not.

In an ideal world, follicular biochemistry and the competence of the corresponding oocyte would be equivalent, which is more likely the situation in litter-bearing mammals such as rodents and rabbits, where the number of newborns

is usually equivalent to the number of follicles that develop in natural cycles. However, this is clearly not the situation in the human, and it has been long known that developmental competence is embryo specific, and more recent evidence demonstrates that this specificity arises in the preovulatory oocyte. This gives reason for optimism that investigations capable of displaying a comprehensive picture of the biochemistry of each follicle's fluid will be informative and clinically beneficial in infertility treatment. This might also suggest new avenues of study related to the site(s) and function of potential competence-determining biomarkers that would increase significantly our understanding of the developmental biology of the human oocyte and the dynamic changes at the nuclear, cytoplasmic, and plasma membrane levels that lead to viability.

Protocols and procedures evolve in science and medicine and clinical IVF will not be exempted from the inevitable forces of change that can be envisaged for this field in the near term. If outcomes are universally improved, then basing the most fundamental of all decisions in clinical IVF, namely, which oocyte to inseminate and which embryo to transfer, on algorithms rather than empirical criteria should be welcomed, even if the long-standing and central role of the human observer is diminished or eliminated.

Review Questions

1. Which follicular fluid components that have been related to competence may be the most informative and easily quantifiable for the typical clinical IVF program?

2. What are the apparent physiological consequences of variability in the expression of angiogenic factors at the transcriptional and translation levels and (i) how might they be detected noninvasively and (ii) be related to oocyte and preimplantation embryo developmental competence?

3. What are the logistical issues that are likely to be associated with the collection and preparation of individual fluid aspirates during the oocyte retrieval phase of an IVF cycle?

4. At present, what highly compelling evidence related to outcome exists that would warrant follicular fluid analysis in routine IVF practice for selective purposes?

5. Going forward in considering the biochemical and molecular complexity of follicular fluid, what other classes of components, such as lipids or bioactive peptides, might be useful to investigate as potential competence biomarkers and why?

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Livestock Production via Micromanipulation

Akira Onishi and Anthony C. F. Perry

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Learning Objectives

- To present the technical aspects of artificial oocyte activation in livestock embryo production
- To learn the engineering of livestock genome by transgenesis
- To describe the xenograft-ICSI technique

We summarize the use of micromanipulation techniques in the production of livestock mammals, focusing on intracytoplasmic sperm injection (ICSI) and the pig, *Sus scrofa*. ICSI is a powerful method for assisted fertilization. It is typically employed where semen characteristics are insufficient for conventional in vitro fertilization (IVF), in which sperm and eggs are mixed and fuse in culture. Unlike IVF, ICSI mechanically delivers the sperm deep inside the egg cytoplasm by injection through a micropipette.

Mammalian ICSI was first demonstrated in hamster oocytes [1] and subsequently applied to humans to overcome impaired fertility [2]. In livestock, ICSI is also used as a procedure for fertilization, but its purpose is not restricted to impaired male fertility. Although circumstances do not ordinarily justify ICSI for breeding normal livestock, there are clear exceptions. Where sperm-containing ejaculates cannot be obtained, a small number of sperm may be obtained by biopsy, with ICSI as the method of choice for delivery into oocyte. Owing to its technical robustness, ICSI is especially beneficial where the breeding male stock has high genetic merit. These applications are now considered in greater detail.

79.1 The Application of Intracytoplasmic Sperm Injection

The potential of ICSI in the conservation of genetic resources, transgenesis, and animal production using sex-sorted spermatozoa has all long been recognized [3]. However, ICSI in livestock animals is less widely applied than it is in humans due to the low success rate coupled to the prohibitive costs involved. This low success rate likely reflects the complexity of interactions between gamete components. Some of these interactions are ectopic, since ICSI introduces sperm plasma and outer acrosomal membranes in addition to acrosomal contents—all components that do not enter the oocyte during natural fertilization. The formation of male pronuclei after ICSI is affected by these ectopic components [4], and to address this, a range of methods have been employed that deplete sperm membranes before ICSI (e.g., [3]). Methods include sperm treatment with triton X-100, dithiothreitol (DTT), progesterone, repeated freezing and thawing without cryoprotectant, or piezo-driven pulses. These methods all damage membranes leading to loss of motility, but the use of *living*, motile spermatozoa is not necessary in delivery by ICSI. The methods that set out to damage membranes are not essential in human and mouse ICSI.

In livestock animals, oocytes are usually removed from ovaries collected at the abattoir and matured in vitro (IVM) because direct collection of sufficient oocyte numbers following maturation in vivo is prohibitively time-consuming and costly. For example, porcine ovulation after hormone treatment yields ~35 oocytes (i.e., in vivo-matured oocytes) per animal at a cost of JP ¥40,000–50,000 (US $455–569 as of July 2010), whereas a single ovary obtained from the abattoir for ~JP ¥150 (US $2) yields 10 oocytes after IVM. Although relatively large numbers of oocytes can be stably sourced via IVM, their developmental potential is probably slightly lower than that of oocytes matured in vivo (Table 79.1). Nevertheless, IVM oocytes support development to term at comparable rates, making them considerably more cost-effective.

79.2 Overcoming Technical Difficulties

Discrepancies between the developmental potential of oocytes derived in vitro and in vivo may also reflect their respective abilities to support physiological oocyte activation, which includes metaphase II (mII) exit and cell cycle progression [5]. In humans and mice, ICSI using sperm from healthy donors is generally sufficient to induce oocyte activation. In livestock animals, additional artificial activation stimuli (parthenogenetic agents) may be required to induce viable embryonic development (Table 79.1), for reasons that are largely unclear. Activation stimuli include electrical pulses, ethanol, calcium ionophore, or specifically ionomycin combined with the protein kinase inhibitor, 6-dimethylaminopurine (DMAP) (Table 79.1). The necessity of supplemental activation factors after ICSI in livestock presumably reflects one or more deficiencies of IVM oocytes, perhaps caused by the failure of injected sperm to trigger release Ca^{2+}, but this cannot always be the case [6, 7], and there is little direct evidence for it. There are no reports of Ca^{2+} release in pig ICSI, but demembranated pig spermatozoa contain the activating factor, phospholipase C zeta, and readily activate mouse eggs leading to pronuclear formation [8]. This suggests that the failure involves a maternal effect, although porcine oocytes can also be induced to undergo Ca^{2+} release [9]. However, unlike mouse oocytes, those of pigs and some other livestock species (including cattle) do not respond to the parthenogenetic agent, $SrCl_2$, so there may be fundamental differences that are not readily explained by differences in the oocyte maturation protocols (e.g., in vitro vs. in vivo or mouse vs. pig). In cattle, there is a single report that ICSI induces abnormal Ca^{2+} oscillations and activation, with the majority of oocytes unable to undergo any Ca^{2+} oscillations at all [10]. These findings seem germane to the low success rates of ICSI in livestock species to date.

Table 79.1 Experience with micromanipulation techniques in farm animal species

Species	Oocytes	Sperm	Activation	No. embryos transferred (recipients)	No. offspring (pregnancies) [% embryos]	References
Pig (*Sus scrofa*)	In vivo	Fresh	Sperm	69 (3)	3 (1) [4.3]	Martin [21]
Pig	In vivo	Fresh	Ca^{2+} ionophore	84 (4)	1 (1) [1.2]	Kolbe and Holtz [22]
Pig	IVM	Frozen	Electrical	16 (1)	1 (1) [6.3]	Lai et al. [23]
Pig	In vivo	FACS	$CaCl_2$	341 (4)	13 (4) [3.8]	Probst and Rath [24]
Pig	IVM	Frozen	Electrical	598 (7)	3 (2) [0.5]	Nakai et al. [25]
Pig	IVM	Frozen	Sperm	452 (6)	1 (2) [0.2]	Yong et al. [6]
Pig	IVM	Fresh	Sperm	197 (7)	12 (3) [6.1]	Katayama et al. [7]
Cattle (*Bos primigenius*)	IVM	Fresh	Sperm	8 (7)	3 + 1?(4) [50.0]	Wei and Fukui [26]
Cattle	IVM	Fresh	Ethanol	nd (10)	5 (5) [–]	Horiuchi et al. [27]
Cattle	IVM	Frozen	Sperm	11 (6)	1 (2) [9.1]	Galli et al. [28]
Cattle	IVM	Frozen	Ethanol	19 (17)	9 (10) [47.4]	Oikawa et al. [29]
Cattle	IVM	Frozen	Ionomycin + DMAP	11 (8)	1 (1) [9.1]	Oikawa et al. [29]
Cattle	IVM	Fresh	Ethanol	61 (54)	24 (28) [39.3]	Horiuchi [30]
Sheep (*Ovis aries*)	IVM	Fresh	Sperm	38 (17)	9 (6) [23.7]	Gomez et al. [31]
Horse (*Equus ferus*)	In vivo	Fresh	Sperm	31(12)	2 (3) [6.5]	Cochran et al. [32]
Goat (*Capra aegagrus*)	No reports					

The retrieval of eggs—or ovum pickup (OPU)—is available for the nonsurgical collection of cattle and goat oocytes matured in vivo. However, with the exceptions of pig and horse (Table 79.1), there are few, if any, reports of ICSI in livestock animals using in vivo-matured oocytes. The application of ICSI in livestock animals has gradually increased, notwithstanding that the efficiency remains low—a source of optimism for those who wish to improve the efficiency further.

79.3 Using Micromanipulation to Engineer Livestock Genomes

One potential prize for such an improvement is the enhancement of transgenesis (the generation of animals with completely or partially prescribed genome alterations). Microinjection of DNA directly into zygotic pronuclei has been used for several years in livestock animals [11, 12], but zygotes (1-cell embryos) are difficult to obtain and manipulate (those of species such as *S. scrofa* are opaque, due to high lipid content), and the efficiency of integration and transgenerational transmission of the foreign DNA remains low [13]. Relatively recently, this problem has been addressed by somatic cell nuclear transfer (NT) (Fig. 79.1). Broadly, somatic cells—for example, ear punch or embryonic fibroblasts—may be cultured and subjected to genetic modification in vitro by transfection with a suitable DNA construct, prior to their use as nucleus donors. This enables the structure and expression level of the resulting integrant to be determined so that the best can be expanded clonally. In this way, donor cell cultures containing ~100% of the desired integrant can be used for NT, yielding high rates of transgenesis [14].

Perhaps the most powerful application of the transfection-NT approach is in the production of gene-targeted livestock animals [15]. In mice, targeted genomic mutagenesis by homologous recombination is widely achieved using embryonic stem (ES) cells, but widely accepted ES cells have not yet been established from livestock animals. The rate of homologous recombination in most somatic cells is typically lower than that in mouse ES cells, with the notable exception of the chicken cell line, DT40 [16]. However, this relative inefficiency may not hold for acutely isolated embryonic cells; gene targeting is efficient in pig embryonic fibroblasts, resulting in the production of gene-knockout pigs by NT (unpublished data). Thus, the transfection-NT combination holds considerable promise in transgenic livestock production.

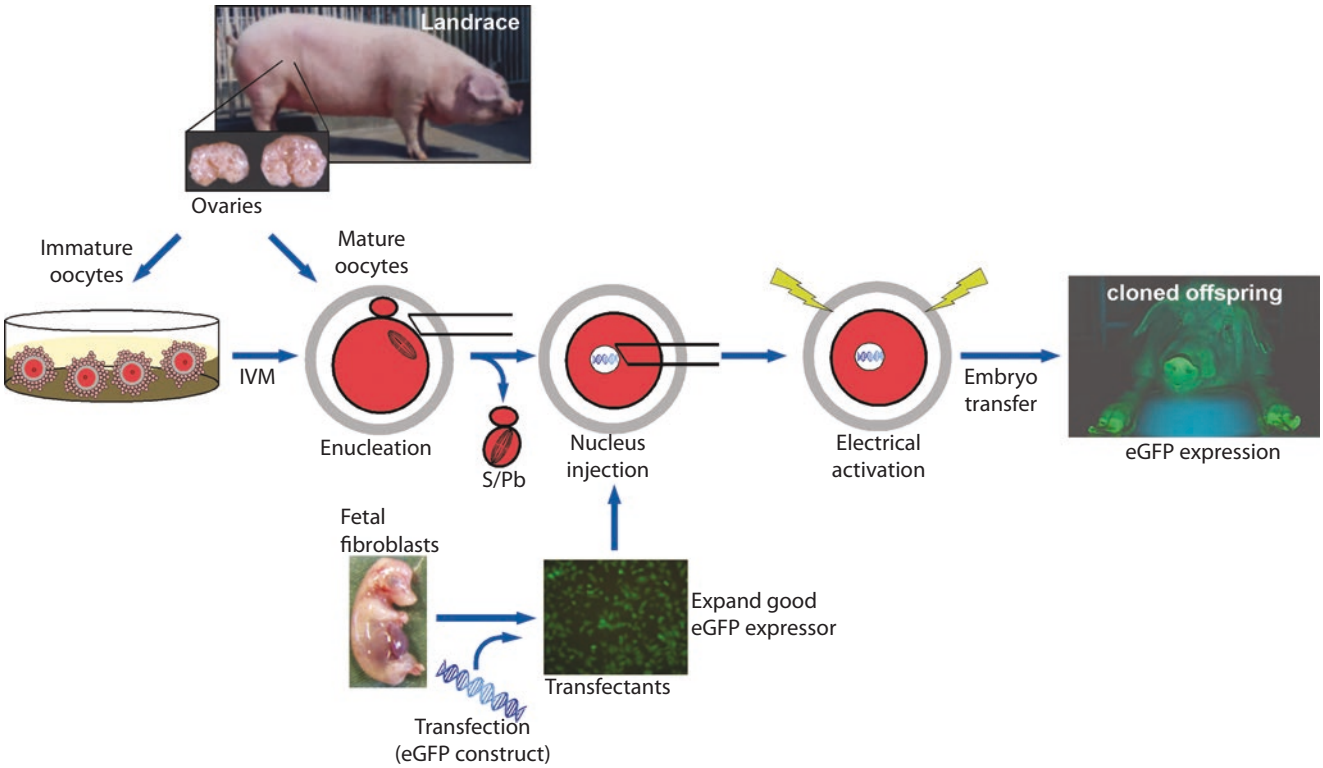

Fig. 79.1 Production of the enhanced green fluorescent protein (eGFP) transgenic pigs by somatic cell nuclear transfer. Fetal fibroblasts were transfected, and cells expressing high levels of eGFP were selected for use as nucleus donors. This method results in cloned pigs that almost always exhibit broad, high-level eGFP expression. *S/Pb* spindle/first polar body

ICSI provides an alternative to transfection-NT for the production of transgenic livestock animals, although, so far, not gene-targeted ones. In this method, spermatozoa are incubated with the transgene DNA construct to form a sperm–DNA complex that is injected into the egg; in effect, the sperm acts as a carrier for the transgene, at least in the mouse [17]. This technique is advantageous in the stable incorporation and expression of large (>100 kb) DNA constructs such as yeast artificial chromosomes (YACs) that are not amenable to transfection or viral delivery [18]. The successful production of nontargeted transgenic pigs following both ICSI and NT techniques has been reported, albeit without using artificial chromosomes [19].

79.4 ICSI in Large Animal Xenografting

Recently, viable piglets have been produced with spermatozoa from immature testicular tissue xenografted into immunodeficient mice [20]. In these experiments, testes from 6- to 12-day-old piglets (i.e., prepubertal pigs) were minced and grafted into the testes of immunodeficient mice. It was possible to collect porcine spermatozoa from the engrafted host mice 133–280 days later and utilize them for porcine ICSI. Although only 6 piglets from 2 recipients in 23 trials were obtained, all grew normally. The xenograft-ICSI technique has several potential applications. It represents one avenue for the conservation of species and other genetic resources and suggests a new means to sustain lineages—especially genetically modified ones—that otherwise propagate with high rates of male neonatal mortality.

Review Questions

1. Describe the methods to overcome the oocyte activation problems in livestock ICSI.
2. Explain the methods to engineer the livestock genome.
3. What are the potential applications of animal xenografting and ICSI?

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Supplementary Information

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