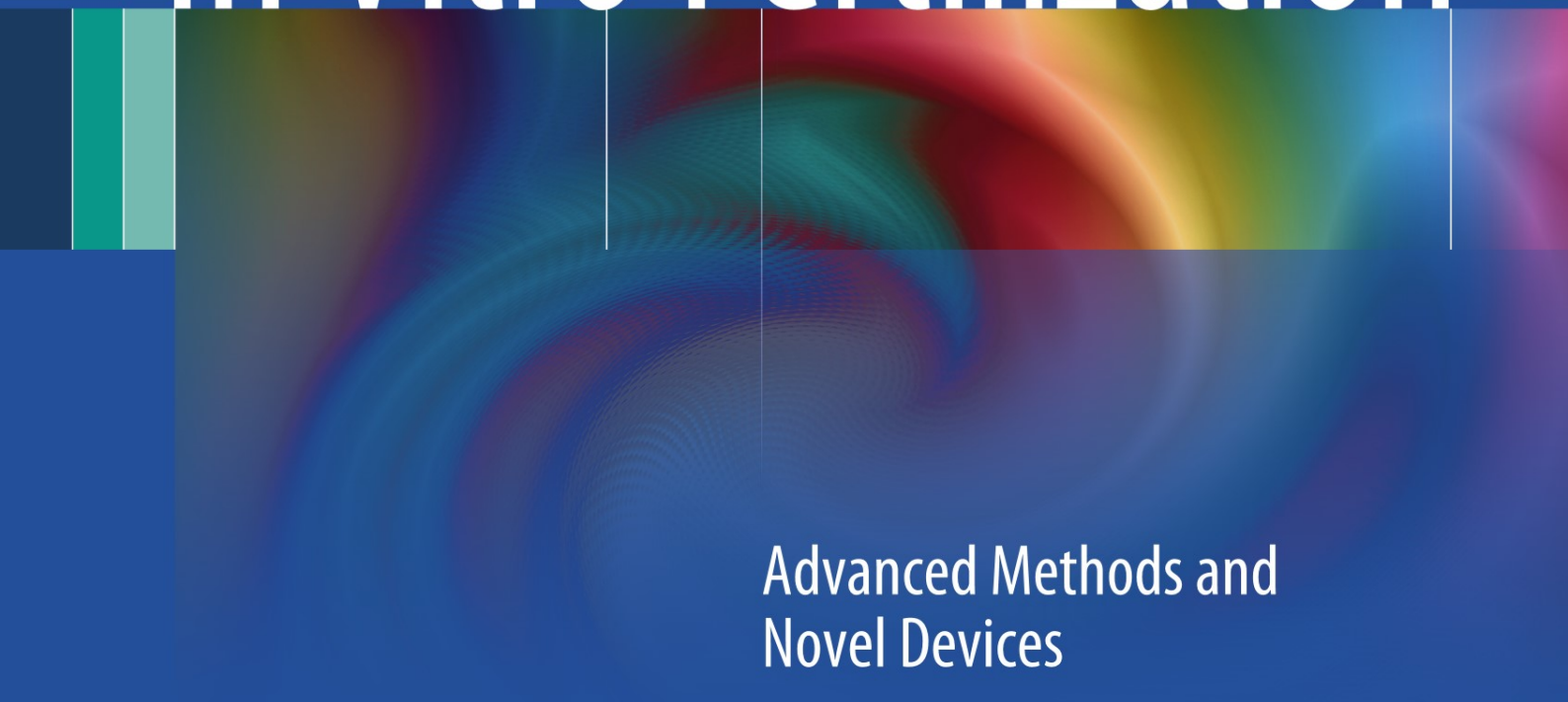
Zsolt Peter Nagy
Alex C. Varghese
Ashok Agarwal
Editors

Practical Manual of In Vitro Fertilization



Advanced Methods and Novel Devices

Springer

Zsolt Peter Nagy • Alex C. Varghese
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Practical Manual of In Vitro Fertilization

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Foreword

Even more than 3 decades after the birth of Louise Brown and the birth of several millions of children conceived by numerous procedures of assisted reproductive technology (ART), the *Practical Manual of In Vitro Fertilization*: *Advanced Methods and Novel Devices* is very welcome. The book is edited by Zsolt Peter Nagy, Alex Varghese, and Ashok Agarwal and consists of more than 70 different chapters written by experts in the field. The authors are mostly from North America, but the book includes some experts from Europe and Australia.

The large numbers of chapters are categorized into different major sections: general organization of ART laboratory, the equipment and culture systems used, the characteristics of the oocytes, different procedures of embryo culture, sperm processing and selection, different insemination procedures, the evaluation and grading of embryos, biopsy of oocytes and embryos, cryopreservation of gametes, embryos and tissues, embryo transfer procedures, accreditation and licensing and legislation in different countries. The last part of the *Manual* consists of a series of special topics.

As is the case in all multiauthor books (in this case more than 70 chapters), it is not surprising that there is some diversity in how the different topics are reported. This is the balance between a textbook by one or two authors and a textbook involving not far from two hundred authors. The *Manual* has its place for all involved in the area of reproductive medicine and biology. It is useful for those novices in the field as for those with years of experience. It is especially focused to the ART laboratory which junior and senior embryologists will find very useful. Since ART requires a multidisciplinary approach to be successful, this book has very useful information for all professionals, including reproductive endocrinologists, counselors, nurses, psychologists, etc.

I am convinced that this *Manual* will be of great value for those involved in ART and will be an important aid for all practitioners.

André Van Steirteghem

Preface

In vitro fertilization (IVF) is the most advanced medical technology for the treatment of infertility. During this process, oocytes from the woman and the sperm from the man are brought together outside of the body, in an "artificial" environment (initially using glass made test tubes or Petri dishes and from which the name of the procedure in vitro originates). The first successful application of this technology was in 1978, marked by the birth of world's first "test-tube baby," Louise Brown. Since then, it is estimated that well over four million babies have been born thanks to IVF and thanks to Patrick Steptoe and Robert Edwards. In recognition for this achievement, the Nobel Prize in Physiology or Medicine in 2010 was awarded to Robert G. Edwards. Since the first breakthrough, there have been several significant discoveries and improvements made related to this technology, helping to increase its efficiency several fold.

This textbook has been written with the aim of providing the most comprehensive update on all laboratory aspects of IVF, both theoretical and practical sides, in great detail. In addition, this book also describes several novel techniques that are currently considered experimental, but that in a few years time may become standard procedures.

A total of 75 chapters are included in this book, focused around the following topics: Setting Up and Running an IVF Laboratory; IVF Laboratory Equipment and Culture Systems; In Vitro Fertilization; Embryo Culture Methods; Sperm Processing and Selection; Insemination Procedures; Micromanipulators and Micromanipulation; Embryo Evaluation, Grading, and Assisted Hatching; Biopsy Procedures on Oocytes and Embryos; Cryopreservation; Embryo Transfer; Management and Regulation in the ART Laboratory; and Special Topics. It is of particular interest that these topics were written by the most acclaimed and acknowledged professionals of our field, 184 in total, representing all continents of the world.

Because of the wide range of topics and the comprehensive theoretical and detailed practical descriptions, this book is an ideal reference for all who are involved with assisted reproduction, including embryologists, andrologists, reproductive endocrinologists, and scientists, regardless if one wishes to obtain a basic understanding or a deep, up-to-date presentation.

We would like to thank Richard Lansing, Executive Editor, for his support and advice and Margaret Burns, Developmental Editor, for her enthusiastic and continuous efforts in reviewing and editing each of the manuscripts. Furthermore, we are thankful to all of the outstanding contributors for sharing their knowledge and for being part of this great project. Finally, we are indebted to our families, who provided their support and understanding when time was taken away from them.

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Journey of Human Gametes In Vitro: 1978–2010

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Zeev Shoham

Abstract

In the 1890s, Walter Heape reported the first known case of embryo transplantation in rabbits. As early as 1932, it was suggested in both fiction and research that human in vitro fertilization (IVF) might be possible. The 1959 birth of rabbits as a result of IVF opened the way to assisted procreation. The field of IVF has developed dramatically during the last 30 years and continues to do so. New drug development, better ovarian stimulation techniques, and improved ways to identify the best embryos will lead to improved pregnancy rates.

Keywords

In vitro fertilization • Ovarian stimulation techniques • Ovarian stimulation protocols • Cryopreservation and storage of oocytes • Embryo transfer

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 [1].

In 1932, Aldous Huxley's *Brave New World* was published. 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 USA, 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 [2] 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 are 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 manuscript 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.

Development of IVF

In 1965, Edwards together with Georgeanna and Jones at Johns Hopkins Hospital in the United States attempted to fertilize human oocytes in vitro [3]. It took another 8 years until the first IVF pregnancy was reported by the Monash research

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team of Professors Carl Wood and John Leeton in Melbourne, Australia. Unfortunately, this resulted in early miscarriage [4]. 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 [5]. However, a breakthrough and a huge achievement was 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 Steptoe and Edwards [6]. Following this achievement, a year later, the first IVF birth was noted in Australia by the joint Victorian Monash-Melbourne team which occurred at Royal Women's Hospital [7], following the announcement of Howard and Georgianna Seegar Jones about the delivery of the first IVF baby in the United States in 1981. This first IVF birth in the United States 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* [8].

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 manuscript will describe the fields which were developed in parallel, enabling the improvement of the pregnancy rate. These fields are as follows:

- Stimulation protocols
- Culture media
- Aspiration technique, handling of the oocyte, and embryo replacement
- Improved fertilization
- Cryopreservation and storage of embryos and oocytes
- Future manipulation of the oocytes and embryos

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. Trounson et al. introduced the use of clomiphene citrate and hMG in the treatment protocols [9]. It was later demonstrated that GnRH agonists can be used to eliminate premature luteinization and control ovarian stimulation [10].

It was Porter et al. who were the first to use GnRH agonists in IVF treatment [11]. Three major protocols using GnRH agonist were developed: the long, short, and ultra-short 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 either 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 [12].

Following the development of the various stimulation protocols, it was noted that there is a possibility that the steroids production during the luteal phase was not optimal, and it was Casper et al. who were the first to describe the use of low-dose hCG for support of the luteal phase in ART cycles [13].

In order to simplify the stimulation protocol and to shorten it, efforts were invested in developing the GnRH antagonist. 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 [14]. 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 by Itskovitz-Eldor et al. [15]. The increasing needs in infertility treatment created the needs to develop new sources for gonadotropins. Recombinant FSH became available during the year 1992, and very soon after, pregnancy was established using a recombinant drug for ovarian stimulation [16, 17]. 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 et al. [18].

The above-mentioned development summarizes 30 years of gradual development in drugs production and methods of administration. Several additional developments were noted when Emperaire et al. publish their observation that the final stage of ovulation induction can be induce by endogenous LH released by the administration of an LHRH agonist after follicular stimulation for IVF [19].

In addition, a new technology was developed which involved in vitro maturation (IVM) of the oocyte, and 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 started cycle [20]. 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.

Culture Media

Optimal culture media which reflects the changing needs of the developing embryo is of importance for success of IVF treatment. The environment of the oviduct/uterus change dramatically as the zygote/embryo travels. The culture media needs 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 [21]. 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 [22]. Gardner introduced sequential media and blastocyst transfer which now greatly assists in the move to single embryo transfer [23, 24].

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 intracytoplasmic sperm injection (ICSI) were introduced in 2000 [25], and since then, the medium remained almost the same.

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 started with laparoscopic retrieval introduced by Steptoe and Edwards in 1978, and laparotomy for infertility related conditions [26].

Lenz and Lauritsen examined and proposed the transabdominal transvesical oocyte aspiration using an ultrasound-guided needle [27]. A year later, Gleicher and his group reported the first vaginal egg retrieval using an abdominal ultrasound [28].

Wikland, together with Hamberger and Nilsson in Gothenburg, Sweden, described the possibility of using a vaginal sector scanner (transvesical or transvaginal technique) for oocyte aspiration [29]. At the same time, Strickler et al. [30] described embryo transfer using abdominal ultrasound guidance. This technique gained additional support from the work done by Feichtinger and Kemeter [31] and it remained the main technique for egg collection until today.

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 [32]. In parallel, the possibility of using the natural environment of the human tube for the process of fertilization and development of the embryo lead to the development of the gamete intrafallopian tube (GIFT) procedure, and it was Asch et al., in 1984, who were the first to report on pregnancy following translaparoscopic GIFT procedure [33].

Much effort was invested trying to improve fertilization when this would not occur naturally when the egg and the sperm where 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 [34] which was followed by the report of a health delivery using the same technique by Ng et al. [35]. Further achievement was noted when pregnancy was obtained from micromanipulation using zona drilling or mechanical partial zona dissection [36]. However, a breakthrough was achieved when Palermo et al. from the group in Brussels reported on the first pregnancy after ICSI [37].

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 anucleate cytoplasm has been used, and Choen et al. [38], was the first to report on a birth after such a procedure.

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 [39]. A few years later, in 1990, Gordyts et al. reported on the first successful human cleavage-stage embryo vitrification followed by a successful delivery [40]. However, until this period of time, there were difficulties to cryopreserved oocytes, and only in 1999, the birth following vitrification of human oocyte was reported by Kuleshova et al. [41]. During the same year, Porcu et al. reported on the first birth from cryopreserved oocytes and

testicular sperm [42]. A major development was noted when Bedaiwy et al. reported in 2006 on the successful cryopreservation of intact human ovary with its vascular pedicle [43]. This was followed in 2008 by the announcement of Porcu et al. on cryopreserved oocytes in cancer patients and the first ever birth of healthy twins after oocyte cryopreservation and bilateral ovariectomy [44].

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, by creating of artificial menstrual cycles, and by using a special hormonal formula for the first 10 weeks of pregnancy [45]. This came along with the first successful delivery following egg donation [46]. Still at the same year, in vitro maturation and -fertilization of morphologically immature human oocytes in an IVF setup were developed and published [47]. 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 Cohen et al. who achieved a blastocyst development from IVM oocyte plus ICSI plus assisted hatching, which was ended in a healthy birth [48].

The role of assisted hatching is still controversial, and the indication for the procedure is not very clear. However, in 1985, Cohen et al. reported in the Lancet about a birth after replacement of hatching blastocyst cryopreserved at the expanded blastocyst stage [49]. 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 [50].

A new era in the field of IVF, i.e., preimplantation genetic diagnosis (PGD) was open with the announcement of Handyside et al. first reported on biopsy of human preimplantation embryos and sexing by DNA amplification [51]. This was followed by the report of pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification [52]. 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 [53].

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 various directions to find solution to those couples who are infertile, to those who wish to become pregnant at an elder age, beyond the boundaries of nature, and to those who, due to medical reasons, need to delay the time of procreation.

British physiologist Robert Edwards, whose work led to the first "test-tube baby", won the 2010 Nobel prize for medicine or physiology. As many as 4 million babies have been born since the first IVF baby in 1978 as a result of the techniques Edwards developed, together with a now-deceased colleague, Patrick Steptoe.

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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Part I

Setting Up and Running an IVF Laboratory

Building the Laboratory

2

Dean E. Morbeck and Marlena Duke

Abstract

This chapter focuses on the critical aspects of building a laboratory in a private practice setting as part of an assisted reproductive technology (ART) clinic. Knowledge of these factors is essential for ART professionals planning a new facility or redesigning an existing one.

Keywords

In vitro fertilization laboratory • Setting up an IVF lab • Laboratory layout for IVF • Construction of an IVF lab • Air quality in an IVF lab

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 cleanroom-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 the European Society of Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (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 will focus 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 system (HVAC). 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 is discussed in Chap. 3 and merits emphasizing the importance of hiring a design-build engineer that specializes in HVAC requirements for cleanroom 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 [3–8].

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Laboratory Layout

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 m 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 also whether the program batches their cycles. 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 most embryologists adhere to is no more than 4 patients per standard CO_2 or triple-gas incubator. This standard does not necessarily apply to the new generation of incubators such as MINC™ and all-in-one microscope/incubator modules. 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, 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 allow 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.

Small IVF Center (<300 Cycles/Year)

Small IVF laboratories are by far the most common since most IVF centers in the United States have one or two physicians who perform 100–150 retrievals each per 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 4–6 "series" per year, with a series lasting 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. An incubator is an essential piece of equipment in the IVF laboratory and thus requires a backup. A second incubator also provides better workflow that allows one to be used as a "working" incubator that is opened frequently during oocyte retrievals and micromanipulation, saving the other incubator for a stable culture environment. As the IVF volume grows, the laboratory can add another full-sized incubator or purchase a small benchtop incubator to function as the working incubator.

In addition to two incubators, the small IVF laboratory requires one embryology and one andrology workstation. The embryologist workstation consists of a dissecting microscope and an inverted microscope with micromanipulators. The most common configurations of these two microscopes are the dissecting scope 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 cleanroom design makes them unnecessary [5].

A typical design for the small laboratory consists of two rooms: one for embryology and the other for cryopreservation equipment and preparation of sperm. The equipment in

the second room includes a centrifuge, compound microscope with ×20 and ×40 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 this 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. 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.

Large IVF Center (>500 Cycles/Year)

Although small IVF laboratories are more common, this past decade has brought about an increase in the number of laboratories that perform >500 cycles/year as well as laboratories that perform >1,000 cycles/year. These large laboratories often serve not only the physicians managing the practice but also 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 two dozen incubators or more may be required to accommodate a center juggling 2,000 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 efficient pace without delays while waiting for a place to perform procedures, approximately one 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 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 PGD 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.

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 in. 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 of 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 cleanroom 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, cleanroom 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 [9–12], though it is not clear if this applies to all lighting in the room or just light emitted from the microscopes [11]. Most IVF laboratories either use incandescent light bulbs or cover fluorescent lights with UV sleeve protectors [4]. All lights and utility connections should be sealed and airtight.

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.

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 [13], 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 [14]. This allows the use of 89% N_2/6% CO_2/5% O_2 air 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. 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 [6].

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, are 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 LN_2 storage dewars. Although low-pressure LN_2 tanks (PSI < 30) may be used for some controlled rate freezers, it is essential to use high-pressure LN_2 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 also pH.

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 has 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 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.

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.

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 digital documentation of embryo quality 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 are used not only for documentation of embryos or oocytes for the EMR, but they also provide

an image that can be displayed in the laboratory for teaching purposes or in a waiting room for the spouse to view during a retrieval or transfer. In this 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, changes are on the horizon 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 [8].

Microfluidics [15] is an example of advancement that will likely find its way into the human IVF laboratory in the next few years. Whether these devices, which provide a more physiologic culture experience for embryos and potentially better outcomes, will dramatically change the way we use incubators is unknown. Introduction of small benchtop incubators during this past decade [14], provide a paradigm shift for both space utilization and gas delivery. Perhaps the most exciting of the changes on the horizon is the expanded use of all-in-one IVF workstations, where microscopes and incubators are combined into one system. Detrimental effects of door openings and handling dishes on temperature and pH fluctuations are thus minimized. It is likely that by the time this book is in print, systems will be on the market that combine microfluidics with controlled all-in-one systems to provide a culture environment far superior and much closer to in vivo conditions. These new systems will present challenges to the design of current laboratories, but once in common use, may be easily incorporated into the design of new laboratories.

New Construction: Validation Before Patient Care

New laboratory construction is analogous to a new car: the plastics, paint, and adhesives are new and give it that "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 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 that 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 Chap. 3), 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 that this sensitivity can be further improved by culturing embryos individually [16]. 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.

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 also by 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 provides 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.

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Air Quality Management

3

Johan Guns, Ronny Janssens, and Martine Vercammen

Abstract

Air quality management in the IVF laboratory is very important. This chapter discusses the current applicable legislation. The qualification process and monitoring to ensure compliance are also discussed. Monitoring basically consists of the enumeration of particles and microorganisms, both in rest and in process. Additionally, the measurement of air flow, pressure difference, temperature, and humidity, as well as the measuring of VOCs, are recommended within ART laboratories. VOCs are natural or synthetic chemicals that can vaporize under normal atmospheric conditions. A negative correlation exists between VOC levels and human embryo development.

Keywords

Air quality management in the IVF lab • IVF lab air quality • Legislation for air quality in the IVF lab • Monitoring microorganisms in the IVF lab • Filtration in the IVF lab

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.

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The current Good Tissue Practice (cGTP) for Human Cell, Tissue, and Cellular and Tissue-Based Products (HCT/Ps) has been enacted by the US Food and Drug Administration (FDA) in 2001; imposing methods, facilities, and control mechanisms for manufacturing HCT/Ps [1]. 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]. Communicable disease transmission through reproductive HCT/Ps has indeed not been reported frequently; either the risk is really low, or transmissions have been underreported [2]. However, 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 [3]. 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

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subsequent microbial inactivation process require EU GMP grade A (ISO 5, class 100) air quality for particle and microbial colony counts, as defined in the European Guide to Good Manufacturing Practice (EU GMP) Annex 1. Additionally the background environment needs to be appropriate for the cells or tissues concerned, at least EU GMP grade D. However, less stringent environmental conditions may be acceptable if the risk of transmitting bacteria or fungi 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 air flow is harmful to the tissue or cells. A grade A environment will be extremely difficult to obtain considering the current ART practices, but also to demonstrate due to technical limitations. The presence of microscopes and CO_2-incubators in the safety cabinet, a high manual working load using many disposables and only a grade D (ISO 8, class 100,000) background environment to support the safety cabinet (ten times less clean than the recommended supporting area by ISO14644-4 [4] and US cGMP [5]) is a challenge. Besides the particle/particulate and microbial content, also the volatile organic composition (VOC) of the air is important since it influences embryo development and clinical pregnancy [6–10].

Qualification and Monitoring

Once the ART establishment has specified its air quality requirements, compliance with the designated classification has to be demonstrated in the formal process of qualification (or validation). The process of qualification demonstrates that the environment and process meet previously defined requirements. Qualification is generally performed on a yearly basis by a testing organization that performs normative (compliant with standards) tests. The ART establishment and the testing organization mutually agree on the type of tests and their scheduling, in this case compliant with the ISO14644-1 and ISO14644-2 standards [11, 12]. Qualification 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. Qualification is followed by monitoring in order to control performance, both in rest state and in operation.

The same techniques can be used for qualification and subsequent 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. 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 and other tissue/cell establishments, derives from pharmaceutical guidelines. The EU directive [3] refers to Annex 1 of the EU GMP [14] that specifies the techniques for particle and microbial testing, similar to those described in the US cGMP [5] and the US Pharmacopeia (USP) [15] for the production of medicines for human use. These pharmaceutical guidelines can guide the ART establishments in the setting up of a monitoring program. However, Europe and the USA apply different classification systems for both air cleanliness (Table 3.1) and

Table 3.1 Air cleanliness classification—USA and Europe

Classification				Maximal number of particles/m³				
ISO14644-1/US cGMP operation	EU GMP at rest	EU GMP operation	USP ⟨1116⟩ operation	ISO14644-1/US cGMP[a] ≥0.5 µm	≥5.0 µm	EU GMP ≥0.5 µm	≥5.0 µm	USP ⟨1116⟩ ≥0.5 µm
ISO 5/100	A B	A	M 3.5	3,520	29	3,520 3,520	20 29	3,530
ISO 6/1,000			M 4.5	35,200	293			35,300
ISO 7/10,000	C	B	M 5.5	352,000	2,930	352,000	2,900	353,000
ISO 8/100,000	D	C	M 6.5	3,520,000	29,300	3,520,000	29,000	3,530,000

[a]US cGMP only specifies particles of more than 0.5 µm

Table 3.2 Recommended limits for viable airborne particulate—USA and Europe

Classification			Maximal number of CFU in the environment				
			US cGMP		EU GMP		USP ⟨1116⟩
US cGMP operation	EU GMP operation	USP ⟨1116⟩ operation	CFU/m³	CFU/4 h (plate diam. 90 mm)	CFU/m³	CFU/4 h (plate diam. 90 mm)	CFU/m³
ISO 5/100	A	M 3.5	1	1	<1	<1	<3
ISO 6/1,000		M 4.5	7	3	–	–	–
ISO 7/10,000	B	M 5.5	10	5	10	5	<20
ISO 8/100,000	C	M 6.5	100	50	100	50	<100
	D	–	–	–	200	100	–

Table 3.3 Recommended limits for microbiological contamination on surfaces—USA and Europe

Classification			Maximal number of CFU on surfaces			
			US cGMP	EU GMP CFU/contact plate (diam. 55 mm)	USP ⟨1116⟩ CFU/contact plate (24–30 cm²)	
US cGMP	EU GMP	USP ⟨1116⟩			Surfaces	Floor
ISO 5/100	A	M 3.5	–	<1	3	3
ISO 6/1,000		M 4.5	–	–	–	–
ISO 7/10,000	B	M 5.5	–	5	5	10
ISO 8/100,000	C	M 6.5	–	25	–	–
	D	–	–	50	–	–

recommended microbiological levels (Tables 3.2 and 3.3). Furthermore, the EU GMP makes a distinction between environmental monitoring at rest state and monitoring of the aseptic process in operation [14]. Environmental monitoring verifies at rest state whether the environment is ready for the forthcoming activity, while aseptic process monitoring aims to ensure that the people, process, and the environment remain in control during operation. The classification limits for microbial monitoring defined by the EU GMP Annex 1 relate to aseptic process monitoring [14].

Practical Implication and Technical Aspects

Monitoring basically consists of the enumeration of particles and microorganisms, both in rest and in process. Additionally, the measurement of air flow, pressure difference, temperature, and humidity, as well as the measuring of VOCs, are recommended within ART laboratories.

Particle Testing

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 mostly situated in aseptic environments (grade A, ISO5, class 100) 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), 1 cfm corresponding to 28.3 L/min, permits qualification and monitoring, although they are not so easy to handle because of weight. 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 grade A environment implies continuous monitoring of particle counts [5, 14]. Since probes have to be positioned within a distance of 12 in. (30 cm) of the exposed product in order to give evidence for aseptic processing, as advised for liquid sterile fill [5] and the need for equipment in ART clean rooms is high, portable or handheld particle counters can be an alternative.

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 (V_s) is not specified, neither in the ISO14644 nor in the European or US GMP guidelines. For qualification however, it is elaborated in the ISO14644-1 guideline [11] as $V_s = (20 / C_{n,m}) \times 1{,}000$. 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, 20/29,300 x 1,000=0.68 L is required in an ISO 8 (GMP grade D at rest) environment, where the maximal concentration for particles larger than 5 µm is 29,300/m³ air. The ISO14644-1 guideline also demands to increase the calculated volume to minimal 2 L within minimally 1 min sampling time [11]. This equation can easily be used for monitoring an ISO 8, ISO 7, and ISO 6 clean zone. However problems arise for an ISO 5 (comparable to GMP grade A) environment. According to the formula, ($(20 / 29) \times 1{,}000 = 690\ L$), 690 L air must be sampled, taking 4 h with a handheld- and 25 min with a portable particle counter, and this for only one sample at one sample location. For time sake one might be tempted to collect only 2 L during only 1 min, as minimally required in the ISO14644-1 guideline for qualification [11]. Doing so induces errors. It is not inconceivable to detect one 5 µm particle using a handheld particle counter with a flow rate of 0.1 cfm in an ART safety cabinet containing a micro-

scope. This corresponds to 354 particles/m³ air, exceeding the class limit of an ISO 5 environment. It is therefore unrealistic from an economic and practical point of view to apply the above for monitoring an ISO 5 environment. The presence of 5 µm particles mostly reflects the occurrence of an event, such as a movement, operational equipment, and infection. We advise to monitor them anyway both at rest and in operation. Even though the measurement will be relatively unreliable due to a too small sample volume, increments will indicate problems. The amount of 0.5 µm particles on the contrary reflects the base state of the clean zone, as determined for instance by the quality of the high efficiency particulate air (HEPA) filter expected to capture particles of 0.5 µm or bigger. The amount of 0.5 µm particles is stable over time and can easily and reliably be quantified with the former formula, resulting in realistic sample volumes because of their higher prevalence. A minimum sampling volume of 5.68 L ($(20/3{,}520)\times 1{,}000 = 5.68\ L$) for monitoring an ISO5 environment can be collected with a handheld and portable particle counter, within 2 min sampling time or less. In conclusion, a practical approach is to measure both 0.5 and 5 µm particles for 2 min and to increase the collection time if 5 µm particles are found.

Sampling Locations

The minimum number of sample locations increases with the process area and can be calculated by the formula $N_L = \sqrt{A}$ where A represents the area in square meters [11]. In case of unidirectional horizontal airflow, the cross section of the moving air perpendicular to de direction of the air flow is considered the area. Locations must be evenly distributed throughout the area and positioned at working height. These guidelines derive from ISO14644-1 guideline and are intended for qualification purposes but we suggest applying them also for monitoring [11]. Additional sampling location could be considered on the basis of a risk analysis in function of ART specific process and product locations.

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 [5, 14]. 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 procedures 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 [16]. In this section, we will discuss how to set up a routine microbial monitoring program for ART clean rooms.

Frequency of Microbiological Monitoring

The monitoring frequency depends on the classification: the lower the maximum permitted particulate the higher 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 [5, 14]. 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 [17]. 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 mircroorganism, 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.

Microbiological Sampling Locations

As discussed, the ISO14644-1 guideline provides a formula for the calculation of the minimum sampling locations for qualification purposes. Guidelines for microbial monitoring such as ISO14698-1, 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 the sampling locations chosen in the initial qualification study 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 [18]. For aseptic process monitoring specific sampling locations must be chosen in function of the process, workflow and/or temporarily incidents.

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 [5, 14, 15]. 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 (CFU) 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 [18–24]. Current air samplers are thought to underestimate bacterial concentrations, especially those of single bacterial cells with 0.5–1.0 mm diameter [24]. Technical innovations are expected to improve precision and sensitivity and might change absolute numbers of detected organisms [15]. The choice of an air sampler can be determined by the validation of the instrument, either by the manufacturer or a third party, in agreement with annex B of the ISO14698-1 that deals with collection efficiency [25]. The cut-off 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 cleanroom-dressed operators is less than 2.1 mm [26].

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, CFU are counted. Results are reported as number of CFU per 4 h exposure time [5, 14]. 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 [27]. The EU GMP and US cGMP advise 4 h exposure time [5, 14]. 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 (TSA) plates with a diameter of 90 mm (Heipha Diagnostika reference 030826e) (unpublished data). Petri dishes were weighted before and after exposure in either a horizontal (flow rate 0.46 m/s) or vertical laminar airflow cabinet (flow rate 0.40 m/s) for different time periods.

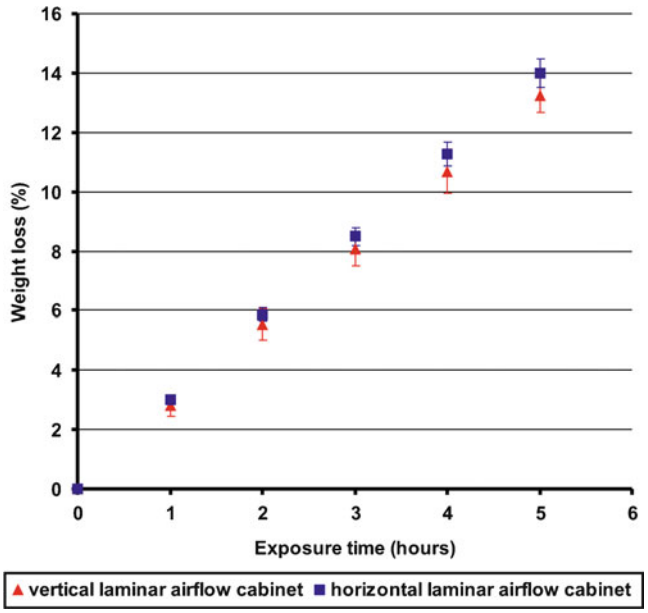

Fig. 3.1 Weight loss of settling plates in laminar airflow cabinets (mean and SD of five measurements)

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 h exposure (Fig. 3.1). A 13% loss in water content has been shown correlated with an 8% loss of viability of test organisms [27], 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 [5, 14].

Monitoring of the Disinfection Program Using Contact Agar Dishes

EU GMP [14] and USP [15] 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 during several seconds, allowing the organisms to adhere to the agar. In case of nonflat surfaces, 24–30 cm² surface has to be swabbed [15] 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 [28]. The EU GMP [14] encourages the combined use of different types of biocides.

Selection of Microbiological Culture Medium

The first international standard for biocontamination control in clean rooms and associated environments ISO14698-1:2003 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 [29]. 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 [30]. Therefore the culture medium has to be appropriately validated for the growth of diverse bacteria and fungi [5]. Both the USP and European Pharmacopoeia (EP) describe several adequate culture media for the sampling and quantification of microorganisms in controlled environments or nonsterile products [15, 31]. 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 [16]. But the USP has now proposed the Soybean-Casein Digest (SCD) Agar or TSA as an all-purpose solid medium for the recovery and quantification of most environmental microorganisms in clean rooms, both bacteria and fungi [15, 16]. 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 EP 2.6 can be used if validated for the purpose intended.

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 [7, 10, 32]. 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 [7]. 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 uncomparable between suppliers. At present no commercial neutralizer is able to inactivate all biocides. Sutton tested six neutralizing broths against 13 commercially available biocides and none of them proved adequate for all tested index organisms (*Trichophyton mentagrophytes*, *Psuedomonas aeruginosa*, *Bacillus subtilus*, *Staphylococcus aureus*, *Clostridium sporogenes*, *Salmonella choleraesuis*, and *Escherichia coli*) [33]. 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, among them the methods of Clontz or Bleisteiner [16, 34]. 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 presence or absence of neutralizing agents. In the method described by Bleisteiner, the same number of CFU is inoculated on three culture plates, two of them supplemented with neutralizing additives, 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% of the growth in the untreated plate supplemented with neutralizing additives.

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, 2–5 days at 30–35°C for bacteria vs. 5–7 days at 20–25°C for fungi and molds [5, 16, 29]. Therefore some centers collect duplicate samples, which doubles sampling time and reagent costs. Alternatively, one general culture medium such as TSA can be incubated under biphasic conditions [30, 35].

In our hands, 7 days of biphasic culture (2 days at 35°C followed by 5 days at 25°C) with TSA medium detected 76–207% of the total CFU detected after 7 days at 35°C (N=3) and 65–100% of the total CFU detected after 14 days of biphasic culture (2 days at 35°C followed by 12 days at 25°C) (unpublished data). Between 2 and 3 weeks biphasic culture (2 days at 35°C followed by 19 days at 25°C) no new CFU were detected. The selected culture condition should detect at least 50–200% CFU detected with the reference method. Our isolates were identified as *Staphylococcus* (*Staphylococcus condimenti*, *Staphylococcus epidermis*, *Staphylococcus capitis*, *Staphylococcus haemoliticus*, *Staphylococcus hominis*, *Staphylococcus aureus*, and *Staphylococcus lugdunensis*), *Corynebacterium* spp., *Dermabacter hominis*, *and Methylobacterium rhodesianum*, comparable to the ones found by Herlong in his ART laboratory [30].

Volatile Organic Compounds

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 report a negative correlation between VOC levels and human embryo development [7]. 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 [6, 7]. Elevated aldehyde concentrations in laboratory air after the use of road sealants to re-surface a parking lot were shown correlated with detrimental mouse embryo development [32]. Emissions from motor vehicles and building renovations influence embryo quality and clinical outcome of ART [32]. 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 gases used for incubators or anesthesia, from plastic Petri dishes, and from cleaning agents or alcohols such as methanol and isopropyl alcohol.

Prevention

The best policy to reduce the level of VOCs in the laboratory air is prevention, starting in the planning phase of design and selection of building materials for their low VOC releasing property [36]. Common products used for furniture such as Formica, pressed wood or fiberboard release formaldehyde and 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 paint formulated for low VOC content should be used for interior painting. If epoxy paints are required for regulatory reasons, low VOC products are advised.

Ideally, the lab is equipped with an over-pressured heating, ventilating, and air-conditioning (HVAC) system with pre-treatment of air with active carbon and oxidation using potassium permanganate and/or photo oxidation 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 [6]. 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 alcohol, isopropanol, and ether. Products for cleaning and disinfection of floors and surfaces require special attention. Alcohol-based detergents should be avoided. Nonembryo toxic 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 a alcohol-free quaternary ammonium compound blended with a biguanide and a alcohol-free amphoteric surfactant for daily floor disinfection. Styrene can be released from polystyrene culture dishes into the air of the incubator [7]. As a preventive measure, disposable culture materials can be removed from their plastic wrapping in a dedicated area, thereby releasing previously accumulated volatile compounds (outgassing). Material used for incubators release VOCs. New incubators can contain high levels of VOCs that should be removed before use [37]. Gases from metal bottles containing compressed gases can contain up to 60 different VOCs, the most obvious being benzene, refrigeration agents, and isopropyl alcohol [37]. Activated carbon and potassium permanganate 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).

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 [7]. Gas chromatography and mass spectrometry of gaseous samples collected in stainless steel sampling canisters [7] permits 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 [38]. The stainless steel vacuum containers are placed in the laboratory for a fixed period of time and send for analysis. Alternatively, chemical sensor badges [39] can be placed in the laboratory for 24 h and send 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 photo ionization detectors (PID) with a broad detection range (0–10,000 ppm) are ideal instruments for detecting sources of VOC contaminations in laboratory air. In our laboratory, we use a fixed continuous VOC detector which can be linked to a permanent monitoring system trough a 4-mA analogue output.

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 [8], while increased pregnancy rates without blastocyst effect were shown in presence of an

intra-incubator air purification unit by Merton [10]. Fertilization rates did not change in either study. VOCs can be effectively removed by solid carbon and potassium permanganate filters [7]. Commercial systems are available for gas bottle inlets, intra-incubator, as well as whole laboratory air purification since filters can either be included in the central air-handling system or in separate stand alone units [40–42]. The efficacy of filter-based stand alone systems in a cleanroom 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 [41, 43]. In existing laboratories without VOC-filtering HVAC systems, stand alone systems can be considered.

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Organizational Aspects of the Laboratory in a Tertiary Care ART Center

4

Nancy L. Bossert and Christopher De Jonge

Abstract

Success in the delivery of the tertiary level health care by an assisted reproductive technology center relies upon the equal partnership between the clinic and the laboratory. It is critical that the laboratory strives to produce the highest quality data for its clinical partners. The laboratory director has the responsibility of ensuring compliance with federal regulations, which were enacted as a framework to improve the quality of testing in clinical laboratories, including the personnel responsible for testing. The success of the assisted reproductive technology center is largely a result of the combined efforts of the clinical and laboratory staff. This chapter will highlight some of the organizational and operational aspects that the laboratory contributes.

Keywords

Assisted reproductive technology center • Organizing an art • Federal regulation in an assisted reproductive technology center • Andrology testing • ART laboratory personnel

"In medicine, tertiary healthcare is specialized consultative care, usually on referral from primary or secondary medical care personnel, by specialists working in a center that has personnel and facilities for special investigation and treatment" [1]. The Fertility Clinic Success Rate and Certification Act of 1992 defines assisted reproductive technology (ART) as all fertility treatments in which both eggs and sperm are handled [2]. By this definition, it is intuitive that patients requiring such assistance would most likely have been referred by a more generalized medical practitioner to the ART center where specialized investigation and treatment can be carried out by personnel specifically trained to provide those services. Thus, ART, by definition is considered tertiary healthcare. This chapter will highlight some of the organizational and operational aspects that the laboratory contributes to support a high functioning and successful ART center.

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Levels of Service in a Tertiary Care ART Center

The need for special investigation and treatment of the infertile couple obligates that ART centers provide several essential laboratory services. Therefore, the list of services that a tertiary level ART center should minimally provide includes andrology, embryology, and phlebotomy. "In-house" endocrine testing as an additional laboratory service is beneficial to all customers for reasons that will be addressed below. Genetic testing has an increasingly important role in ART service provision and, as a consequence, it is becoming a laboratory fixture in many ART centers.

Complementing the expansion of laboratory services within the ART center are additional services that have gained increased importance for the on-site delivery of comprehensive care to the infertile couple, and these are urology, psychology, and, to an extent, research. The integration of these services into an ART center's scope of care adds significant value to the patient for reasons that will be discussed below.

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Andrology

Andrology tests range in their complexity from moderate to high, depending on whether qualitative or quantitative assessments are being made [3, 4]. To accommodate the tertiary level investigation and therapy necessary for the infertile couple (e.g., traditional semen analysis and specimen processing for intrauterine insemination or in vitro fertilization [IVF]), the andrology testing performed will by definition be classified as high-complexity. The types of specimens to be analyzed or processed will typically be diverse and consist of fresh ejaculates produced antegrade and/or retrograde, cryopreserved partner or donor ejaculates, epididymal sperm aspirations, and testicular biopsies. The latter two specimen types will be supplied by an in-house or referring physician with specialized training for those procedures who works in close association with the ART laboratory and clinic. Semen cryopreservation and anti-sperm antibody testing are other andrology services that may typically be performed at an ART center.

Procedures for andrology testing and quality control can be as diverse as the resources used to obtain them, and this has, in general, proved problematic for this field [5]. Adherence to standardized methods enables test results to be universally shared and interpreted between clinics. Further, to comply with federal law, laboratories must participate in proficiency testing, and the use of common methods allows for greater power when evaluating laboratory performance relative to peers. The recommended source for standardized andrology laboratory procedures and quality assurance is the "WHO laboratory manual for the examination and processing of human semen, 5th ed" [6].

Embryology

In contrast to the andrology laboratory, the embryology laboratory remains unclassified in terms of its CLIA '88 (Clinical Laboratory Improvement Amendments of 1988) complexity [7]. It has been 10 years since the American Association of Bioanalysts (AAB) failed in their attempt to force the federal government to rule on embryology laboratories. Since then there have been notable changes in the embryology laboratory, e.g., embryo biopsy for preimplantation genetic diagnosis, so perhaps the time is ripe to lobby the federal government to seriously reconsider applying the CLIA '88 classification scheme.

By definition, an ART laboratory should be able to offer as part of its service provision: oocyte retrieval, oocyte insemination, micromanipulation techniques such as intracytoplasmic sperm injection (ICSI) and assisted hatching, blastocyst culture, embryo transfer, and embryo cryopreservation. In comparison to the aforementioned laboratory activities, preimplantation genetic diagnosis/screening (PGD/S) is a relative newcomer as a routine service in the clinical embryology laboratory. Most embryology laboratories will limit their technical involvement in PGD/S to only performing the embryo biopsy. The biopsy specimens will then be shipped to a referral laboratory for molecular analysis while the biopsied embryos remain in culture awaiting diagnosis. Because the time from biopsy to diagnosis typically requires extended embryo culture (5–6 days total culture) a laboratory must be able to demonstrate that it has the ability and proficiency to perform the culture. The cost-effectiveness of in-house PGD (biopsy + genetic analysis) must be seriously considered before expending the personnel and financial resources.

Another embryology laboratory service that an ART center might provide is oocyte cryopreservation. Oocyte cryopreservation is considered by some to be an experimental technique that should only be performed using an investigational protocol under the auspices of an internal review board (IRB) [8]. That said, this service has already gone the route of donor sperm whereby one can use the internet to find an "egg vendor" and then flip through the directory of egg donors to find a suitable match. In addition to the commercialization of this service, egg freezing, in general, is becoming more and more common in ART centers.

Phlebotomy/Endocrinology

A tertiary level ART center should be able to provide the timely reporting of serum female hormones such as estradiol, progesterone, follicle-stimulating hormone, and human chorionic gonadotropin. Most phlebotomy and endocrinology services in an ART center are classified as low to moderate complexity largely due to the history of automation in the field. The phlebotomy service should be performed by an individual who has been specifically trained to perform venipuncture. The phlebotomist functions on the frontlines in an ART center, oftentimes being the first person the patient will encounter in multiple visits over a short period of time. Thus, the phlebotomist is a key customer relations point person; oftentimes assuming the role as the patient's sounding board for complaints, anxieties, and other emotional issues. The phlebotomist then is extremely valuable in setting the tenor of the patient's subsequent experiences at the center.

Once the blood specimen is collected and processed, the determination of serum hormones can be done in-house or by a reference laboratory. For a stand-alone center, the lease or purchase of an analyzer allows for greater control and independence in delivering care to its patients. University- and hospital-based ART centers may be required to use the clinical laboratory testing service. While hospital-based

service may provide high-quality results, they may be somewhat limited in their flexibility to accommodate unique circumstances that are part of an ART service.

Psychology

The importance of psychology as an on-site service in the ART center has increased dramatically over the years in parallel with the increased incidence of surrogacy, donor gametes and embryos, same-sex patients, etc. Many ART centers require patients undergoing IVF or using third-party reproduction (donor gametes, surrogacy, gestational carrier) to pass psychological screening prior to commencing treatment. While on the one hand, the psychologist serves as a compass for patients with regard to gamete source and the other aforementioned pregnancy-related issues, they also serve a useful role for more routine aspects, such as coping mechanisms for stressors associated with hormone fluctuations, financial concerns, and negative outcomes.

Urology

Urology services are necessary when there is a suspected male factor. With specialized training in male infertility, these essential partners provide critical diagnostic service, and they contribute to therapeutic requirements, such as the need for a testicular biopsy or epididymal sperm aspiration. A number of centers in the United States have forged very successful on-site collaborations between urology and reproductive endocrinology services. University-based ART centers oftentimes have an easier ability to develop such a relationship. In doing so, the delivery of health care to the infertile couple can truly be a "one-stop-shopping" experience.

Research

In addition to the above-named clinical services, some ART centers have active basic and clinical science research programs. Research is not an absolute necessity in an ART center, but it can significantly augment the knowledge base within the program and the field as well as provide the opportunity to practice evidence-based medicine.

Essential for supporting a successful research program is accurate and reliable information flow among relevant parties both intra- and extramurally. A failure to provide informed consent or a deviation from clinical protocol could have severe consequences for the research program and, potentially, the ART center.

Laboratory Personnel

Personnel Qualifications

CLIA '88 was established in 1988 and implemented in 1992 as a United States federal measure to improve the quality of testing in all clinical laboratories. Whether a laboratory is classified as moderate or high complexity, CLIA '88 specifies regulations for all laboratory personnel, including laboratory director, technical supervisor, and testing personnel.

The specific federal requirements for personnel performing andrology testing can be found in the Federal Register [2]. Briefly, laboratories performing high complexity testing procedures such as andrology testing are required to have a qualified laboratory director, technical supervisor, clinical consultant, general supervisor, and testing personnel. A qualified laboratory director may assume more than one of the above-mentioned positions. In order to be qualified to manage and direct the laboratory personnel and performance of high complexity tests, the laboratory director must:

1. Possess a current license (if required) as a laboratory director in the state in which the laboratory is located and.
2. Either be a doctor of medicine, osteopathy, or podiatric medicine licensed to practice medicine, osteopathy, or podiatry in the state in which the laboratory is located.
   (a) Be certified in anatomic or clinical pathology (or both) by the American Board of Pathology or the American Osteopathic Board of Pathology or possess qualifications that are equivalent to those required for such certification or.
   (b) Have at least 1 year of laboratory training during medical residency (e.g., physicians certified in hematology by the American Board of Internal Medicine) or.
   (c) Have at least 2 years of experience directing or supervising high complexity testing.
3. Or hold an earned doctoral degree in a chemical, physical, biological, or clinical laboratory science from an accredited institution and.
   (a) Be certified and continue to be certified by a board approved by Health and Human Services or.
   (b) Prior to February 24, 2003, must have served or be serving as a director of a laboratory performing high complexity testing and must have at least 2 years of laboratory training or experience (or both) and at least 2 years of laboratory experience directing or supervising high complexity testing.
4. Or prior to February 28, 1992, be a laboratory director who previously qualified or could have qualified as a laboratory director under regulations at 42 CFR 493.1415 published March 14, 1990.
5. Or prior to February 28, 1992, be an individual qualified under state law to direct a laboratory in the state.

In addition to the CLIA '88 regulations, the Practice Committee of the American Society for Reproductive Medicine (ASRM) published and then revised their guidelines for human embryology and andrology laboratories [9]. While the functional and operational aspects of these guidelines relative to the andrology laboratory precisely mirror the regulations of CLIA '88, there are some rather significant differences, specifically as they relate to the embryology laboratory, that make this document essential for the laboratory library.

CLIA '88 regulations do not apply to the embryology laboratory, so there is no designation for testing complexity and, as a consequence, they are not subject to the same federal regulations as the andrology laboratory. However, in spite of the lack of federal regulations, ASRM set forth guidelines for embryology laboratory personnel, including laboratory directors. According to ASRM, a qualified embryology laboratory director must:

1. Hold an earned doctorate degree (Ph.D.) from an accredited institution in a chemical, physical, or biological science as the major subject *or* hold a medical degree (M.D. or D.O.) from an accredited institution *or* have qualified as a laboratory director prior to July 20, 1999. Effective January 1, 2006, all new laboratory directors should hold High Complexity Laboratory Director (HCLD) or American Board of Bioanalysis Embryology Laboratory Director (ABB-ELD) certification or its equivalent.
2. Have 2 years of documented experience in a program performing IVF-related procedures; This experience should include (a) familiarity with laboratory quality control, inspection, and accreditation procedures; and (b) detailed knowledge of cell culture and ART and andrology procedures performed in mammalian systems.
3. Have had a period of training of at least 6 months and have completed at least 60 ART procedures under supervision.

The CLIA '88 qualifications for technical supervisors and testing personnel in andrology laboratories were annotated elegantly by Keel [4] and will not be reiterated here. ASRM guidelines do not specifically address qualifications for technical supervisors in andrology laboratories and merely refer to CLIA '88. For embryology laboratories, ASRM guidelines list qualifications for a "supervisor" without using the CLIA '88 labels of "technical supervisor" or "general supervisor." According to ASRM, a qualified embryology laboratory supervisor should:

1. Meet the ASRM qualification requirements for laboratory director or.
2. Have earned a bachelor's or master's degree in chemical, physical, biological, medical technology, clinical, or reproductive laboratory science from an accredited institution *and* have documented training including a minimum of at least 60 ART procedures under supervision.

ASRM guidelines for testing personnel in andrology laboratories mirror those regulations set forth in CLIA '88. For embryology laboratories, ASRM guidelines refer to "technologists" instead of the CLIA '88 defined "testing personnel." According to ASRM, a qualified embryology laboratory technologist should:

1. Meet the ASRM qualification requirements for laboratory supervisor or.
2. Have earned a bachelor's or master's degree in chemical, physical, biological, medical technology, clinical, or reproductive laboratory science from an accredited institution *and* have documented training including a minimum of at least 30 ART procedures under supervision.

The requirements defined above for laboratory personnel provide a necessary framework that an ART center can utilize when hiring staff. However, the qualifications listed do not speak to the equally important practical aspects of the technical expertise a given candidate may possess. So, just because a potential hire may have the appropriate credentials doesn't ensure that they possess the "right stuff" to excel in the ART laboratory.

Occupational Training

Staff to be hired for the ART laboratory may have received laboratory skills training from a clinical laboratory technologist/technician program or a basic science program at a college or university. For the clinical laboratory technologist or technician, phlebotomy, manual cell counting, and the operation of some automated analyzers should be somewhat routine. However, possession of the same skill set is not a reasonable expectation for the basic science department graduate. Regardless of the type of formal institutional training, most individuals will likely require specific structured in-house training for andrology and ART since neither of these disciplines are a part of the educational mainstream. To address this disconnect, several academic medical centers and universities now offer graduate level training and graduate degree programs that have focused training in andrology and ART, e.g., Cleveland Clinic, University of Kentucky, and the Eastern Virginia Medical School.

There arguably is considerable heterogeneity between ART laboratories and the procedures they use whether in the andrology or embryology laboratory. Therefore, the abilities and expectations of new staff with prior ART laboratory experience are best evaluated after a period of in-house skills competency testing. For example, how was the new hire trained to perform semen analysis? Were they trained to strictly follow WHO guidelines or some variation from that standard? In our experience, it is not uncommon for the "trained" ART technologist to be naïve to hemocytometer use for sperm concentration determination. While on the

surface these differences in technical experience may be superficial, they may portend of underlying technical competency. Thus, it is highly advisable with any new recruit to have a balanced and structured competency assessment plan in place on their first day of work in order to determine fulfillment in the promise of their integration into the ART laboratory.

Laboratory Staffing Guidelines

CLIA '88 states that "the laboratory director must employ a sufficient number of laboratory personnel with the appropriate education and either experience or training to provide appropriate consultation, properly supervise, and accurately perform tests and report test results in accordance with the personnel responsibilities described in this subpart" [3]. CLIA '88 offers *no* guidance on laboratory staffing. In contrast, ASRM has published revised minimum standards for practices offering ART in which they set forth specific guidelines for the recommended number of staff in an embryology laboratory [10]. However, this professional group provides no similar recommendations for any of the other disciplines that are a part of the ART laboratory. The only recommendation is, "The director must ensure that the laboratory has sufficient number of qualified personnel to perform testing."

Perhaps the most critical factors when determining the (in)adequacy of laboratory staffing are quality and safety: quality in the testing being performed and safety of the individual(s) involved in the testing. To simultaneously satisfy these two criteria, it is generally preferred that a technologist handle only one specimen at a time. Thus, based on supply (e.g., blood-draw patients, semen samples, cumulus removal) and using the single specimen at a time principle, one can directly calculate the personnel demand in terms of FTE's (full time equivalents) to assure quality in the delivery of service to all stakeholders. A helpful resource for making these determinations can be found in the publication, "Defining the typical work environment for assisted reproductive technology laboratories in the United States" [11].

Inherently, people work at different levels of efficiency. So, where one staff member may be able to handle a high volume of phlebotomy over a given time frame, that same person may function quite differently when performing other ART laboratory functions. Therefore, when staffing the laboratory the decision should be made whether staff will perform single or multiple functions. If the latter is required, then recognition by the laboratory director of diversity in work efficiency within an individual and between individuals becomes all the more critical when making determinations regarding staffing needs.

Hiring/Firing

Hiring is an exciting time for all. To balance and focus that excitement, it is helpful for the interviewer(s) to have a formalized series of probing questions for the hiring interview to gain some understanding of the candidate's emotional and professional maturity. For example, how does the person cope under stressful situations, how do they view their responsibility when mistakes occur? We have found it helpful to have two senior interviewers, e.g., laboratory director and assistant director or supervisor, for the interview so that a broader appreciation of the candidate can be gained by questioning from different perspectives. It is also helpful to document the proceedings regardless of final disposition, i.e., hire/no hire. Finally, it is important to leave no ambiguity regarding hours and days of work, salary, training requirements, and other critical expectations, e.g., time off policy.

Firing is an emotionally disrupting time for all. Typically the events that lead up to dismissal will have occurred over a period of time rather than from a single catastrophic event. There should be evidence of remedial training if indicated, conflict resolution through mediation, or other interventions to facilitate remediation of the issue(s) at hand. It is critical that there is accurate documentation of the events that have lead up to termination. If there is Human Resources support, it is advisable to work closely together so that all interests are protected. During this time, it is likely that the entire laboratory team will feel tension. Further, the staff member who is following a path toward dismissal may try to recruit other laboratory and/or clinical team members as allies. This can lead to a very divisive environment. Sensitivity of the laboratory and clinic atmosphere as well as the verbal and nonverbal communication by all staff must be closely monitored for contamination. The laboratory director and indeed all of the stakeholders in the general well-being of the ART center must be proactive to prevent such situations from occurring and stop them if they occur.

The Fully Integrated ART Center

The ART laboratory can be viewed philosophically either as an ancillary service, merely providing support to the clinical service provision, or as an equal and essential partner of a fully integrated ART center. Ultimately, it will be the director of the ART center who will make that determination.

Leadership

Leadership will determine the degree to which the ART center will succeed in terms of not only patient satisfaction but also team satisfaction. The age old adage of "lead by

example" is apropos, and it starts at the highest level. A leader who nurtures an environment of respect, open communication, and tolerance will earn followers of the center's mission, which contributes directly to results and patient satisfaction. Lack of respect breeds a laissez-faire attitude. Lack of open communication instills negativity and paranoia. Intolerance creates hostility.

Team

"A team comprises a group of people… linked in a common purpose. Teams are especially appropriate for conducting tasks that are high in complexity and have many interdependent subtasks. A group in itself does not necessarily constitute a team. Teams normally have members with complementary skills and generate synergy through a coordinated effort which allows each member to maximize his or her strengths and minimize his or her weaknesses" [12]. This description is most appropriate as applied to the fully integrated ART center. Referring back to the beginning of this chapter, ART, by virtue of the specialized healthcare it delivers, is considered a tertiary level service. The ART center then is where specialized investigation and treatment can be carried out by personnel specifically trained to provide those services. So, it is essential that ART personnel carrying out these diverse interdependent tasks be acutely aware of their common purpose—the delivery of high quality care to their patients. Operating with that purpose contributes to a fully functional and effective team. It should be the daily mission of leadership to nurture "team."

Summary

Success in the delivery of the tertiary level health care by an ART center relies upon the equal partnership between the clinic and the laboratory. Since approximately 70% of medical decisions are based on laboratory data, it is critical that the laboratory strives to produce the highest quality data for their clinical partners. The federal government enacted CLIA '88 as a framework to improve the quality of testing in all clinical laboratories. The laboratory director has the responsibility of ensuring compliance with CLIA '88 and this includes the personnel responsible for testing. The laboratory staff along with the clinical staff are the heart and soul of the ART center, and the success of the center is largely a result of their combined efforts.

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Quality Control Management

5

William R. Boone and H. Lee Higdon III

Abstract

The cornerstone of assisted reproductive technology (ART) is based upon quality control management. From instruments used to formulate media to methods to determine environmental properties such as temperature, relative humidity, and gas concentrations, all should be monitored and calibrated according to manufacturer's specifications to insure their accuracy. In addition, attention to detail and the will to examine and improve our ART laboratory processes must be our main goals in our quest to provide a high quality, predictable product to the patients we serve. This review discusses quality control and quality assurance as they pertain to the ART laboratories.

Keywords

Assisted reproductive technology • Quality control in the ART laboratory • Quality assurance in the ART laboratory • Air quality in the ART laboratory

In the United States, personnel in assisted reproductive technology (ART) laboratories have to adhere to quality control (QC) and quality assurance (QA) requirements and guidelines set forth in the Clinical Laboratory Improvement Act of 1988. However, individuals in each ART laboratory must adapt these requirements and guidelines to best suit their needs. We will attempt to cover pertinent QC and QA topics as they relate to in vitro production (IVP). These topics will include laboratory environment, culture media, and contact materials.

Quality Control/Quality Assurance

Quality control and quality assurance are two distinct activities. Quality control is any activity performed to ensure procedures and equipment operate according to manufacturer's specifications. For example, the job of laboratory personnel is to insure that equipment will produce the same result every time and within specification. On the other hand, quality assurance is considered to be a comprehensive method to monitor and evaluate the entire analytical process that takes place within the laboratory. Thus, QA is designed to evaluate quality such as accuracy of reports, outcomes, and patient satisfaction [1].

Laboratory Environment

Air Quality

While air quality is discussed in detail in another chapter, a short synopsis is warranted. In this chapter, air quality is divided into three parts—air exchange, particle counts, and volatile organic compounds (VOCs).

Air Exchange

The number of air exchanges can vary greatly among laboratories. Normal air exchanges for operating rooms in South Carolina hospitals are 15 per hour; [2] this number can be

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used as a guideline for ART laboratories. However, if the goal is to approach an ISO 5 (Class 100) rating, then air exchanges probably will have to be increased to reduce particle counts so as to reach ISO 5 compliance. We had to increase our anteroom air exchange rate to 32.8 exchanges/h (31.8 m^3/min) and our clean room, which obtains its air from the anteroom, to 21.2 exchanges/h (12.4 m^3/min) [3]. The number of air exchanges produced can be easily calculated by a professional certifying agency. However, if one knows cubic feet per minute of air exiting all ducts in the laboratory and one knows total cubic feet of the room, it is a simple matter for one to divide cubic feet per minute into cubic feet of the room and multiply by 60 min.

Particle Counts

Because bacteria and other contaminants can attach themselves to particles, a decrease in particles equates to an increase in air quality. While particle counts may not be directly related to fertilization or pregnancy outcome, there are some indirect data to indicate that as laboratory air becomes cleaner (reduced particle counts) there is improvement in ART outcome [3]. If high-efficiency particulate air (HEPA) or ultralow penetration air (ULPA) filters are added to the heating, ventilating, and air conditioning (HVAC) systems, particle counts can be reduced.

Volatile Organic Compounds

VOCs have been linked to reduced outcomes in ART [4, 5]. Aromatic hydrocarbons (benzene, toluene, and xylene), alcohols (ethanol, propanol, and phenol), alkanes (propane and hexane), and aldehydes (nonanal and decanal) are VOCs that can be found in dyes, paint thinners, printing solutions, flues, perfumes, and flavorings used in food products.

To protect gametes and embryos from VOCs in the laboratory, HVAC systems can be equipped with filters imbedded with charcoal and potassium permanganate. (Charcoal traps such compounds as benzene and formaldehyde, while potassium permanganate oxidizes alcohols and ketones). Furthermore, oil overlays of culture media can act as sinks to capture VOCs because VOCs are oil soluble [6].

Temperature

Temperature only a few degrees below core body temperature appears to have little effect on overall embryo survival if exposure time is limited [7]. However, some scientists have observed that even limited decrease in temperature can alter the cytoskeleton of the oocyte [8], spindle fibers [8–10], and other organelles [11, 12].

On the other hand, temperature only a few degrees above core body temperature may have detrimental effects. Elevated temperatures increase the number of polar bodies formed [13] and reduce oocyte competence and embryo development [14]. Ali and associates demonstrated that as outside environmental temperature increased, fertilization rate from intracytoplasmic sperm injection, but in vitro fertilization rate was reduced [15]. In addition, more zygotes were arrested, a lower blastomere number occurred, and embryo quality declined [15]. While pregnancy rate in hotter months was similar to pregnancy rates in cooler months, the abortion rate increased during the hotter months [15].

While some data would indicate that a few degrees shift from normal body temperature may not alter embryonic development, we have demonstrated that a few tenths of a degree can alter human embryo development. Incubators that fluctuated above 37°C (average core body temperature) by as little as 0.3°C lowered pregnancy rate [16].

Incubators are not the only instruments that need to be inspected to insure that temperatures are kept within acceptable ranges. Unlike incubators, which keep temperatures fairly constant provided doors are not opened, temperatures of warming trays and microscope stages fluctuate with the laboratory environment. If room temperature increases, equipment temperature increases; if room temperature decreases, equipment temperature does likewise.

Air current from HVAC systems can alter the surface temperature of equipment by several degrees. If air current is sporadic, the surface temperature of equipment will fluctuate dramatically. One way to reduce this shift in surface temperature is to deflect the exhaust air so it will not blow directly onto the surfaces of microscopes and warming trays. Having stated this, laboratory personnel should make sure that equipment that requires a regulated surface temperature is not located juxtaposed to a refrigerator or other equipment that has an exhaust fan that may alter air current.

Temperature of culture medium should be determined with calibrated probes in a control drop of medium. This should provide a measure of the temperature to which embryos are exposed. Because heating coils do not provide an even temperature on the surface of laboratory equipment, the control drop of culture medium should be placed over the hottest point on the surface, and the temperature of the equipment should be set so that the hottest point never exceeds a temperature of 37°C for human IVP.

Light

It has been known for more than 40 years that florescent lighting is deleterious to cells [17]. More specifically, ultraviolet rays from fluorescent lights damage cells [17, 18]. However, fluorescent lighting is not the only light source that can harm cells. Yamauchi and coworkers demonstrated that incandescent lights can be harmful to golden hamster oocytes [19], while others have demonstrated that neon

lights impaired cell proliferation in two-cell and morula rabbit embryos [11, 12]. Furthermore, Okano and associates demonstrated that photooxidation can produce hydrogen peroxide, which can alter DNA in cells [20]. This can occur in as little as 0.5 min [21].

However, not all scientists agree with the above findings. Some scientists have reported no damage to mouse oocytes [22] or embryos [23] after exposure to fluorescent lighting. Likewise, Jackson and Kiessling exposed mouse oocytes to light from a microscope without detriment to future fertilization or development [24]. Furthermore, Bielanski and Hare demonstrated that cattle morulae were not altered when exposed to helium/neon light [25].

Why so much disagreement? This incongruity may be caused by different developmental stages or species of embryos or it may simply be that only certain wavelengths of light alter the development of cells. It seems that light in the color range of blue and below (<490 nm) is most deleterious to oocytes and embryos [26, 27]. This lack of agreement on what causes cells to deteriorate may even be related to the duration of light exposure [28, 29].

While results of cellular development after exposure to light are somewhat conflicting, we would recommend that laboratory personnel limit the amount of exposure that gametes and embryos have to light. With the use of plasticware for IVP comes a reduced exposure to light waves because plasticware will absorb almost all light waves below 300 nm [30, 31]. In addition, laboratory personnel can place green filters on microscope lights to reduce exposure to shorter wavelengths of light. Furthermore, laboratory personnel can have fluorescent lights replaced with yellow lights, or they can have plastic, ultraviolet light-protective sleeves placed over fluorescent light [3]. The amount of light exposure even could be as simple as laboratory personnel lowering light intensity in the room and on the microscope [32].

Relative Humidity

Expressed in percent, relative humidity is the amount of moisture in the air as compared to the maximum amount of moisture that the air will hold at the same temperature. Relative humidity within the incubator has limited consequence if the culture media are covered in oil. The oil overlay appears to block dehydration and thus prevents an increase in osmolality that is harmful to cells. If, however, laboratory personnel still use an open culture system whereby media are exposed directly to the surrounding air, then a relative humidity as close to 100% as possible inside the incubator should be the goal.

Because we use an oil overlay system, our biggest concern with relative humidity becomes conditions outside the incubator. We attempt to maintain relative humidity within the laboratory between 30 and 60%. When the moisture drops much below 30%, static electricity becomes an issue, while increased moisture much above 60% can lead to development of bacteria and mold [33].

Electromagnetic Fields

Even though there are some preliminary results that would indicate that electromagnetic fields (EMFs) can be deleterious to the in vitro culture environment, they have received little study. Sperm exposed to alternating polarity from magnets appeared to have reduced penetration potential [34]. After exposure to EMFs, 50% of late morulae and blastocyst stage mouse embryos failed to develop further [35]. Zusman and coworkers also demonstrated a reduction in crown-rump length and somites in 10.5-day-old rat embryos [35]. Until further research is conducted, we would recommend that cells be kept as far away from an electrical source as possible. Furthermore, it has been recommended that Mu metal (a conglomerate of nickel, iron, copper, and molybdenum) be placed inside incubators to shield cells from EMFs [36].

Culture Media

Hydrogen Ion Concentration

Because hydrogen ion concentration (pH) is so important to embryo development, it is essential that an appropriate environmental pH be provided for the embryo to flourish. While the ultimate method to determine proper pH for embryo development would be to measure pH within the embryo (pH_i), this is not practical. Therefore, the next best method would be to measure pH of the culture medium in which the embryo is bathed. One must keep in mind that pH in bicarbonate-based media (the media used to culture eutherian-mammal embryos) is controlled by the amount of CO_2 that is dissolved in the media. This CO_2 dissolution is determined by partial pressure of the CO_2, which decreases as elevation increases from sea level.

While initial research on mammalian embryos was conducted with 5% CO_2, today's media often are built around 6% CO_2, which drives down pH of the culture media. This 1% increase in CO_2 has been reported to increase blastocyst cell numbers, implantation rate, and fetal developmental rate among mice [37].

What is the proper pH to culture mammalian embryos? While this question has initiated much research, the answer still remains elusive. The proper pH may vary with the species and cell stage of the embryo. For example, eight-cell

hamster embryos thrive better in a 10% CO_2 environment than the usual 5% level [38]. On the other hand, Zhao and associates reported that two-cell mouse embryos will develop in environments containing between 0.4 and 5.0% CO_2 [39]. To add to the dilemma, Plante and coworkers cultured bovine embryos and discovered that "fair" quality embryos developed significantly better at 7.7 pH than at 7.1–7.4 pH [40].

Regardless of this diversity, in general, as the embryo migrates through the female reproductive tract, pH appears to decrease (rat—8.04 in the ampullary region to 7.21 in the uterus [41]; cattle—5% CO_2 produced more fertilized oocytes than did 7%, but 7% produced the best developmental results [16]). John and Kiessling [42] confirmed this with their data that indicated that pronuclear mouse embryos had a higher blastocyst rate when cultured in media at 7.17–7.37 pH vs. 7.5 pH.

Once medium is made, its pH can still shift. Laboratory personnel who make culture media should be aware that water quality and the filtration procedure can alter the pH of media [43]. More importantly, if pH of the medium falls outside acceptable range, it should be discarded rather than adjusted with acids or bases. Even if medium has been properly made, equilibrated, and overlaid with oil, its pH still can shift if exposed to the atmosphere, which contains only 0.035% CO_2 [44]. Therefore, Lane and coworkers recommend that buffers such as *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) or 3-(*N*-morpholino) propanesulfonic acid (MOPS) be added to culture medium to help maintain pH if exposed to the atmosphere [37].

Oxygen Tension

While studied extensively, an appropriate oxygen (O_2) level to be used in an in vitro culture system has yet to be determined. Currently, the two most popular O_2 levels are 5 and 20–21% (the amount of O_2 in the atmosphere). We do know that O_2 can be converted to superoxide radicals that are deleterious to cells [45]. Therefore, it would appear that the less O_2 available, the less opportunity to damage gametes and embryos through the production of radicals. Couple that with the fact that the O_2 level inside the oviduct has been determined to be low (2–8% [46–48]) and the best level for O_2 within the incubator appears to be ~5%. There are numerous reports that appear to validate the use of low O_2 tension to culture various mammalian embryos in vitro (mouse [45, 49–51], hamster [52], rat [53], rabbit [54], sheep [55], pig [56], cow [57], rhesus monkey [58], and human [49]).

However, there also appears to be support for those scientists who use atmospheric O_2 levels (IVP and culture of bovine embryos [14]; culture of follicular cells [59]). Byatt-Smith and associates [60] suggested that at the 5% level, embryos may become hypoxic. However, others would suggest that either level (5 or 20–21%) performs equally well (cryopreserved/thawed human embryos [49]; fertilization rate, blastocyst rate, or pregnancy rate [61]).

Osmolality

Just like pH, a wide range of osmolality can be tolerated by gametes and embryos. This does not mean, however, that osmolality is not critical and does not need to be determined. It can be ascertained easily with a freezing-point depression osmometer. Like pH, osmolality measurements give laboratory personnel a sense of the accuracy with which the medium was prepared. If the medium falls outside of an acceptable range, it should be discarded rather than tweaked to bring it into compliance.

If medium is left for an extended time, it should be rechecked to insure that evaporation has not increased the osmolality. Lane and coworkers demonstrated that 3.0 mL of culture medium left out at room temperature for as little as 30 min caused osmolality to rise by 5%, and if left for 1 h, osmolality rose by 14% [37].

The ideal osmolality has yet to be determined, as it appears to depend upon cell stage and embryo quality [40]. Like gas concentration and pH, osmolality even may be species dependent.

Water Quality

Water may be considered the most important ingredient in culture media. We provided a historical perspective on the use of water for culture media in a previous article [62]. It is sufficient to say that the purer the water, the better the culture media will be. There are now systems available that can provide highly purified water with the use of carbon filtration and reverse osmosis in addition to the water passing through a carbon cartridge, an ion exchange resin cartridge, an organic extraction resin cartridge, a pyrogen-extraction cartridge for polishing, and a 0.22-μm filter for sterilization. Even after water passes through these cartridges, it should be tested for bacterial endotoxins to insure that the level remains below 1.5 pg/mL [63].

Oil Overlay

Previously, we stated that oil is often layered over culture media. Benefits of an oil overlay include reduced media evaporation, heat loss, pH shift, and protection from outside contaminants. Oil overlays even can extract harmful elements that may reside in the culture media [64–66]. However, this same oil overlay may contain contaminants that inhibit

embryo development [43, 67–69]. To reduce potential contaminants in oil and to prevent extraction of nutrients in culture media, media should be washed with various salt and protein solutions before use [64, 66, 70–72].

Volume of Culture Medium

The amount of culture medium to be used for in vitro fertilization should be based upon at least three factors—type of culture vessel used, sperm density, and whether embryos are cultured singly or in groups. While IVP is most commonly performed in petri dishes, other vessels such as culture tubes also have been used [73]. Dinnie and associates demonstrated that concentration of motile sperm played a more important role in fertilization than did total number of sperm present [74]. However, Cunningham and coworkers stated that an increase in sperm concentration can reduce the pH because of an increase in CO_2 production from sperm metabolism [75]. As for group culture, it has been proven to be superior to single embryo culture for cell stage obtained, total number of embryos that reach a specific stage, blastomere numbers, and embryo quality [76–82].

While the volume of culture medium may be as large as 3.0 mL [83] or as small as 1.5 μL [84], the current trend is towards a smaller volume. This lower volume has been linked to higher fertilization rates [84–86] and higher quality embryos [77–79, 84, 87]. It has been suggested that the reason for improved embryo quality is because of autocrine and paracrine factors produced by each embryo and the ability of commingled embryos to "cross talk" in a small volume of medium [84, 88].

Contact Materials

Throughout the last half century, different equipment have been used to make culture media, collect gametes, culture oocytes and embryos, and transfer the final product back to a patient or recipient. For a detailed review of various contact materials, please see our previous article [62]. Suffice it to say, the petri dish and all of its variations have been the primary choice for the culture of oocytes and embryos.

Unfortunately, some IVP devices have been shown to have toxic effects on gametes and embryos. Therefore, toxicity testing has been put into place to help avoid future mishaps. Such toxicity testing includes the mouse embryo bioassay (MEB), the sperm viability bioassay, and other cell assays (e.g., Chinese hamster ovary cells, human blastocyst cells, human amniotic fluid cells [89], mouse hybridoma cells [90], and human luteinized granulosa cells [91]).

Of these bioassays, the MEB appears to be the predominant choice of laboratory personnel, even though it is far from being a perfect test. There is much variation within this assay—the beginning cell stage and the ending cell stage of the bioassay, the mouse strain used, the number of embryos commingled in culture, the volume of media used, and the use of protein in the media—all of which contribute to the sensitivity and accuracy of the bioassay [62]. While the MEB maybe the most popular bioassay, a much more sensitive one is needed.

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Daily, Weekly, and Regular Preparations for the IVF Laboratory

6

Michael A. Britt and Klaus E. Wiemer

Abstract

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. 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.

Keywords

IVF laboratory standards • Standards in the IVF laboratory • Cryopreservation room in the IVF lab • Safety in the IVF laboratory • HVAC system in the embryology laboratory • Gas room in the IVF laboratory

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 and recorded. 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.

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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 separate rooms based upon primary function such as the cryopreservation/cryostorage room, main embryology laboratory, and media preparation room. A separate room for andrology-related procedures is a viable option if space permits. The main laboratory should serve as designated space where all general embryology procedures take place and would house the majority of lab equipment. The media preparation room is designed specifically for refrigeration of media, preparation of culture media, the weighing of chemicals, and general laboratory preparations. The cryopreservation/cryostorage room is utilized for gamete/embryo freezing, as well as short- or long-term cryostorage of embryos and oocytes. In addition to these three main areas of the embryology laboratory, two other areas of high importance are the gas manifold room and HVAC room. This chapter will be organized based on the daily, weekly, and regular preparations required in

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these different sections of the clinical embryology laboratory. Establishment of standards will be discussed as well.

Cryopreservation/Cryostorage Room

The advantage of having a separate cryopreservation room is that oocyte 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.

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.

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. 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 personal in case of a tank failure or drop in liquid nitrogen level below established minimums. The callback 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 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.

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.

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 kept safely for 7–10 days.

Main Laboratory

The advantage to 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, isolettes, antivibration tables, inverted light microscopes with and without micromanipulation capabilities, audiovisual equipment, and incubators.

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. Incubator settings which 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. 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 antivibration 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 plastic ware [3].

The following miscellaneous tasks should be performed on a weekly basis: refill liquid dispensers, change water in incubator pans, 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 of 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 gasses within the incubator as well as test the pH of new media before embryos are introduced to the new lot of culture media. 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 callback system on all incubators, having maintenance performed on all microscopes, cleaning of all incubators, and changing all gas line or incubator carbon filters if applicable. Laboratory floors and surfaces areas should be cleaned on a regular basis as well.

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 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, 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 days 0–6 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 microdroplets 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. This integral aspect of quality assurance helps to ensure that the embryos are never exposed to temperatures that could induce spindle defects, poor developmental rates, or have other deleterious effects.

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. Some typical items that could be housed within the media preparation room include a laminar flow hood in which media 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 the 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.

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 room temperature, the performance of the clean water system if applicable, and 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 make certain 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]. 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 was prepared, and expiration dates for both aliquoted and prepared media with protein. An inventory system for all chemicals used as well as a system of safe disposal of all expired chemicals should be created.

Gas Room

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.

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]. 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 depend 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 semiannually.

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.

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Risk and Safety in the IVF Clinic

7

Peter Sjoblom and Julius Hreinsson

Abstract

Prioritising is an important part of risk management as it helps us to allocate resources to where they are of most utility. This requires us to quantify the different risks. All severe risks must be addressed as they can threaten the continued existence of the clinic or the life of a patient. However, also insignificant events that are highly likely to occur should be addressed, as they will drain the resources of the clinic. This process only indicates the priorities but does not suggest how to address the risk issue. In assessing risk, we are often hampered by the lack of knowledge of the exact nature of the risks, and quantifying the risk of rare events is problematic. For example, our knowledge of long-term risks of IVF is limited because human IVF has not been practised long enough for data to accumulate on this issue. Also, when assessing outcomes, one must know what to look for, and in many cases, we have a limited knowledge of the processes in human development and their vulnerabilities to our in vitro systems and the endocrine environment we create in ART. In principle, there are three ways of managing risk: elimination, reduction and transfer.

Keywords

Risk management in the IVF clinic • Safety in the IVF clinic • Risk identification • Long-term risks of IVF • Ergonomics in the IVF laboratory

Risk Management

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.

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Risk and Conflicts Between Interests

There are inherent conflicts of interest between the 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 recently demonstrated in Italy, where the legislature severely restricted the practice of reproductive medicine after public reactions to claims of human

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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 surplus 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 organisation. Risk management is about balancing risks to different stakeholders, i.e., patients, offspring, community, the profession, owners and staff.

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 non-financial (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.

Assessing Risk

Prioritising is an important part of risk management as it helps us to allocate resources to where they are of most utility. This requires us to quantify the different risks. Risks can be characterised along two dimensions or components, probability and severity, and can be illustrated in a graph with two axes, as shown in Fig. 7.1. The number of steps on each axis can be varied.

All severe risks must be addressed as they can threaten the continued existence of the clinic or the life of a patient. However, also insignificant events that are highly likely to occur should be addressed, as they will drain the resources of the clinic. This process only indicates the priorities but does not suggest how to address the risk issue.

In assessing risk, we are often hampered by the lack of knowledge of the exact nature of the risks, and quantifying the risk of rare events is problematic. For example, our knowledge of long-term risks of IVF is limited because human IVF has not been practised long enough for there to be data on this issue. Also, when assessing outcomes, one must know what to look for, and in many cases, we have a limited knowledge of the processes in human development and their vulnerabilities to our in vitro systems and the endocrine environment we create in ART. While it is clear that IVF is not associated with gross morphological abnormalities, it is not implausible that there might be subtle physiological changes [4, 5].

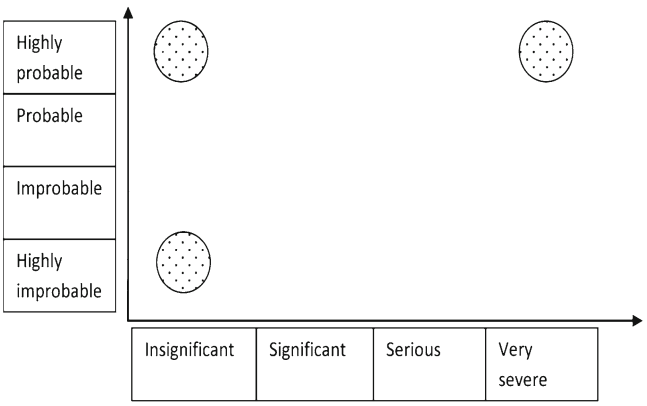

Fig. 7.1 Two risks dimensions: probability and severity. The *top right corner* indicates the greatest risk, and the *lower left*, the lowest risk

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 [6], 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. 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 could increase the likelihood, or compound the effect of an error later in the process.

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.

Risk Tolerance

Since we cannot eliminate risk but only reduce it, we have to decide how far we want 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., optimised.

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 re-designed, 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 re-design 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 [7]. Any non-conformity in this area, even if it is discovered before any damage has occurred, must be recorded and treated as a serious incident, and analysed 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 non-conformance events. Figure 7.2 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.

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 labelled, because we are working with the wrong information or because we misread the information or because we accidentally labelled 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.

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.

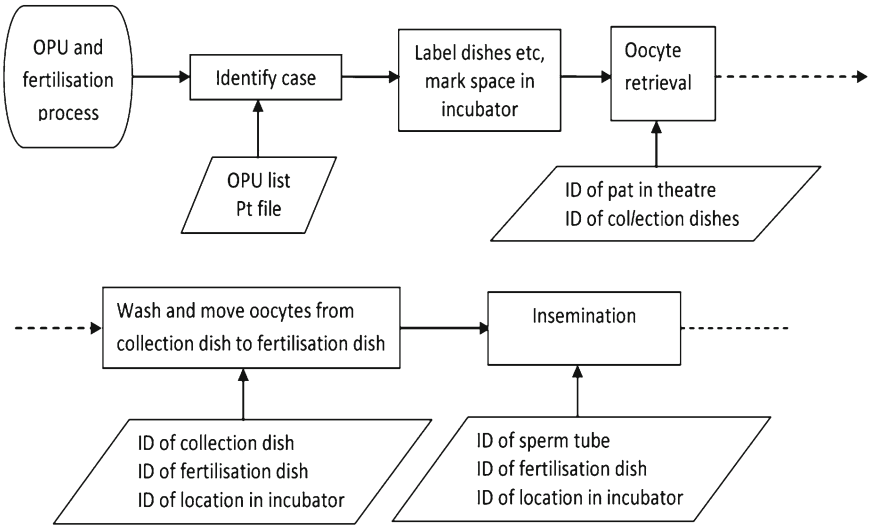

Fig. 7.2 An example of a simple process map for OPU (each step requires elaboration) to assist in risk management of the process. *OPU* ovum pick up

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 work stations 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 at what cost. Today, RFID 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.

Monitoring

When the procedure is being performed, the operator should certify that all the steps have been carried out according the SOP. Witnessing should be certified, electronically or by handwritten signatures. The certification should be audited regularly, and all non-conformities, 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 analysed regularly and system improvements initiated. Training and education may also need to be improved.

Analysing 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. 7.3 is by no means comprehensive.

Critical Decisions Made in the IVF Laboratory

Two decisions, which are critical to the outcome of the treatment, are made in the IVF laboratory: (1) choice of fertilisation method and (2) selection of embryos for transfer and cryopreservation. The consideration in the first case is the risk of fertilisation failure vs. 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.

Fertilisation Method

The use of ICSI varies tremendously between countries, from 35 to 97% [8]. Certainly, the choice between IVF and ICSI is not always easy for the IVF laboratory, and in many cases, a drift towards an increasing rate of ICSI can be seen. It is, however, well established that ICSI improves outcomes only in cases of severe sperm abnormalities or previous fertilisation failure in IVF and is not indicated in cases of female pathologies such as poor ovarian response or in cases of previous implantation failure [1]. 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 fertilisation method based on the clinics patient population, culture methods and, of course, on the quality of the sperm

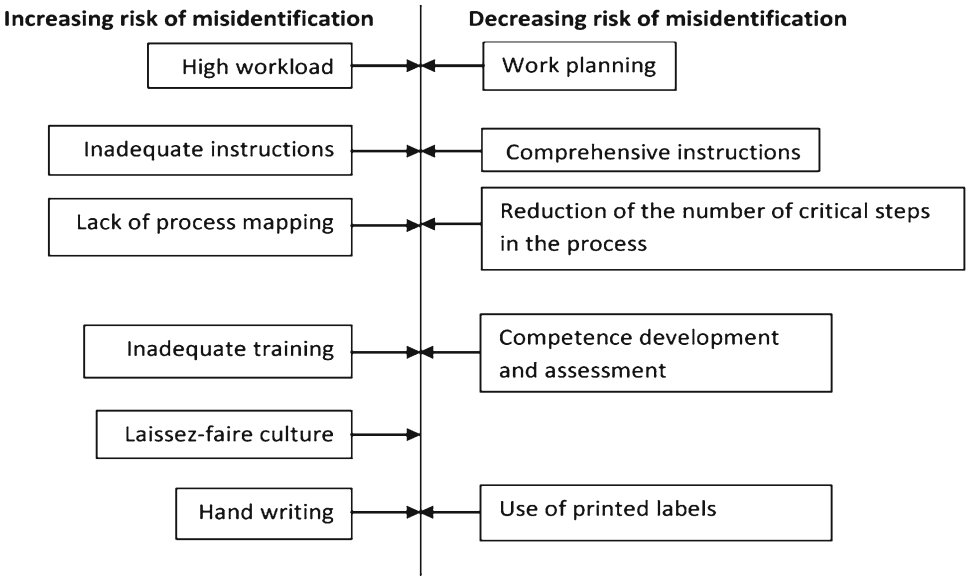

Fig. 7.3 One such approach to identifying factors that increase or decrease risk is force-field analysis, whereby factors or forces that increase or reduce risk are identified and ranked. The example shown is by no means comprehensive

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—non-existent parameters cannot be improved. It is important to realise two aspects of this problem:

- Fertilisation 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 fertilisation failure after IVF in spite of a normal sperm sample have a greatly reduced chance of success with ICSI compared to ICSI for male factor patients [9].

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 fertilisation rate after IVF or ICSI and benchmark them against leading clinics. Over time, this will assist clinics to improve and fine-tune selection criteria.

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.

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, minimising 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.

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 risk to IVF laboratory staff [10]. A review of the health risks associated with LN_2 handling in ART laboratories has been published by Tomlinson [11], 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 above-mentioned risks. Protective clothing and shoes is warranted, especially when handling LN_2 in pressurised 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 litre 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 per cent 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.

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:

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 pipetters and multi-dispensers to minimise 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.

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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Part II

IVF Laboratory Equipment and Culture Systems

Essential Instruments and Disposable Supplies for an IVF Laboratory

8

Leslie Weikert, Christa Fralick, and Klaus E. Wiemer

Abstract

This chapter is intended to serve as a guide in selecting the necessary equipment and disposable supplies required to start an in vitro fertilization (IVF) laboratory. For a new lab, it is 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. 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 quality control program will insure that the proper quantity and quality of materials are on hand at all times.

Keywords

IVF laboratory instruments • Instruments for the IVF laboratory • Microscopes • Incubators • Cryopreservation and storage equipment

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, the 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. If possible, a layout of equipment prior to placing air exhaust vents can help reduce potentially deleterious airflow around microscopes and incubators.

Equipment

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 with micromanipulators. 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. Depending on the laboratory workload, it may be useful to have multiple dissecting microscopes available. If the laboratory offers

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preimplantation genetic diagnosis (PGD), an additional dissecting microscope located outside the IVF laboratory is recommended for cell fixation. 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]. 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 which 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.

Incubators

The primary incubator still in use in most IVF laboratories is the traditional CO_2 incubator which allows one to select the CO_2 concentration with the O_2 level being that of ambient air (approximately 21%). However, there is a trend toward culture in a reduced oxygen environment of 5% O_2, and these models are available in many forms from various manufacturers. These CO_2 incubators use nitrogen gas to reduce the oxygen level. There is also a newer compact benchtop incubator available which uses a heated chamber base plate and lid, providing a 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 of temperature and pH due to the compact chamber, unlike the large upright chambers of traditional water- and air-jacketed incubators. 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.

A gas analyzer is used to monitor the actual gas content within an incubator. 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 have pH at 7.2–7.4. 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 setting of an incubator.

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 which enable you 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 which 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 and require the embryologist to stand throughout the tasks at hand.

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 command for such procedures as insemination of oocytes, change of oocytes/embryos to new dishes, and embryo biopsy cases. 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 suggested to provide each patient with a picture of their embryos 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.

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 day 5 and day 6 blastocysts. Historically, controlled-rate freezers have been used for cryopreservation of early stage embryos and more recently oocytes. Until a reliable method of vitrification of oocytes and cleavage stage embryos is developed, a controlled-rate freezer will continue to be a necessary piece of equipment in the IVF laboratory. Currently, controlled-rate freezers use either alcohol or liquid nitrogen to cool samples. The equipment needed for most vitrification protocols is minimal but varies depending on the protocol used. In order to ensure clear legible writing on cryostraws and vials, a labeling device 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 small 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.

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.

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 back up electrical generators that provide electricity in the event of a power outage. Generator output should be determined after the total amount of equipment that needs to be functional during power outages has 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 on emergency power.

Miscellaneous Equipment

It is generally accepted today that air quality is integral part of an IVF laboratory in consistently producing 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 higher-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.

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 which 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 should be selected that can keep culture media and other important materials within their ranges of temperature for storage. 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.

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; biannual proficiency testing includes this as a required test for culture media. An analytical balance could be used to weigh out chemicals used for making media for various procedures.

Disposable Supplies

Plasticware

Disposable plasticware is utilized extensively throughout the IVF laboratory for everything from oocyte/embryo culture to storage of various solutions. Regardless of intended use, each lab should develop an inventory system in order to insure an adequate supply is on hand. 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 [2] for a period of time after removing from packaging. These compounds are highly soluble in oil and can affect embryo development. 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).

Plasticware for embryo culture is available from many different manufacturers (BD Falcon™, Nunc™, and Corning™) and is a matter of personal choice based on the type of culture system the laboratory director chooses 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 large surface areas 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. This dish size 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.

Plastic dishes for embryo culture tend to be smaller and range in size from 35 to 60 mm. Selection of dish manufacturer is purely a personal choice. Multiwell dishes are used for embryo culture as well.

Oocyte Retrieval Needles

The type and size of oocyte retrieval needles in use today are a matter of choice. 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.

Miscellaneous Disposables

Other materials required for a lab to run efficiently are items such as instruments to move oocytes and embryos, filters for solutions, and supplies for cryopreservation such as canes and goblets. These materials are very dependent on how the lab director manages the lab, number of staff available to perform procedures, as well as budget.

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 as well as 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 insure that the proper quantity and quality of materials are on hand at all times.

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Co_2 and Low-O_2 Incubators

9

Marius Meintjes

Abstract

Embryo incubators can be considered the heart of any in vitro fertilization (IVF) laboratory. Understanding the strengths and weaknesses of our incubators is invaluable and essential to optimize clinical IVF outcomes. Based on the evidence, we should culture in a reduced O_2 environment if possible and, therefore, give preference to incubators that have the ability to provide a reduced-O_2 environment. Second- and third-generation top-load mini-incubators are becoming a significant alternative to conventional incubators, mainly due to the considerably smaller footprint, lower cost of operation, and superior temperature and gas-phase recovery times. However, we should be aware of the unique safety and quality control challenges posed by top-load incubators. When using conventional incubators, those equipped with infrared CO_2 sensors are superior. When having to use incubators with thermoconductivity CO_2 sensors, humidity control is essential and clinical outcomes can be improved by limiting the number of patients per incubator and the number of incubator door openings. The quality of incubator supply gases should be considered as important as the general laboratory air quality and filtered similarly for particles and gaseous contaminants. Compressed N_2 as a source to lower incubator O_2 concentration should be avoided. Small direct-heat air-jacketed incubators are preferred over large water-jacketed incubators. Critical incubator performance parameters should be monitored daily with independent measuring devices. There should be no less than two incubators in any facility, regardless of type of incubator or the patient volume. Incubators must be connected to a 24-h alarm and notification system, monitoring all critical incubator parameters. 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.

Keywords

CO_2 • O_2 • Reduced oxygen • Incubator • In vitro fertilization • Embryo • Blastocyst • Human • Quality control

Embryo incubators can be considered the heart of any in vitro fertilization (IVF) laboratory. Understanding the strengths and weaknesses of our incubators are 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 market place 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

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to review potential costly infrastructure demands, depending on the type of incubator under consideration. The historical standard is 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 bench-top 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.

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 culture in low O_2 concentrations markedly improve in vitro embryo development and subsequent pregnancy outcomes when compared with similar culture 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] are frequently not observed. The human studies which 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 7 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 provide 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.

Bench-Top (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. Bench-top 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, or the constant recirculation of custom-mixed gases through the microchambers of the K-Systems G-185 (Table 9.1), it is understandable that the targeted O_2 and CO_2 concentrations are reached much faster in bench-top

Table 9.1 A comparison of some features of common top-load bench-top incubators

Bench-top 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	$4,600	1×2, 1×4	–	–
K-Systems G-185	Built-in gas mixer	No	$32,000	10×2	+	+

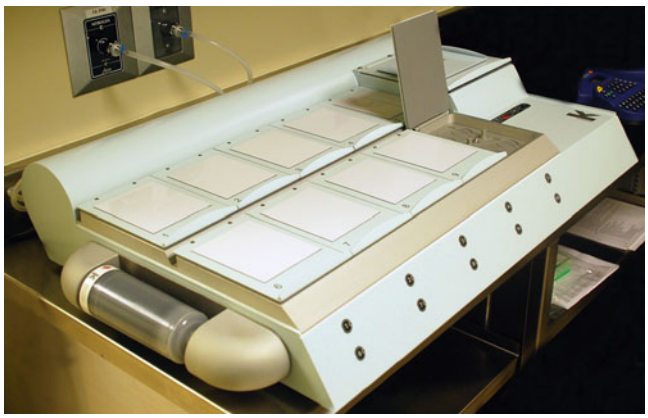

Fig. 9.1 An example of a bench-top, top-load incubator, allowing for direct heat transfer to culture dishes from all sides, fast gas-phase recovery, and in-line N_2 and CO_2 filtration

incubators. The smaller footprint size of bench-top incubators and patient-specific chambers allow for use in confined spaces with minimal disturbance of the culture environment when accessing multiple culture dishes (Fig. 9.1). The minute amounts of gas needed to stabilize a bench-top microchamber and the relative lack of moving parts result in significant savings in maintenance and gas supply, estimated to be in excess of $1,000 per incubator per year. With a single gas source now lasting longer, the gas supply become a lesser source of variation over time. Bench-top 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.

Bench-top 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 opportunity exist to redeem patient material, even with a timely alarm signal. Unlike conventional water-jacketed incubators which gradually lose temperature (~1°C/h) and gas content over time, the destabilizing effect of an untoward event in a microchamber, bench-top incubator is immediate. The BT37 bench-top incubator has 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 bench-top incubators due to the small chamber size and relative inaccessibility of the microchambers. 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 bench-top 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 bench-top 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 use premixed gas only, which make 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, if desired, 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. Media changes every 48 h renders 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 bench-top 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.

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.

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, bench-top 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. 9.2) can have a faster CO_2 recovery time than a smaller incubator with a thermoconductivity CO_2 sensor, e.g., the 170L Sanyo 18M has a faster recovery time than the 49L Sanyo 5M (Table 9.2). Smaller air-jacketed incubators have a smaller footprint and, due to their light weight, can be stacked and incorporated into integrated work stations. Most of the larger incubators (>150 L) were originally designed for bulk cell-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 184L 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.

Fig. 9.2 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

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

Table 9.2 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 18M	IR	Zirconium	Passive	Air	170	–	–	+	Cu-enriched stainless steel
Sanyo MCO 5M	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

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 affect 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 49L Sanyo MCO 5M incubator, only available with a TC sensor, has a longer CO_2 recovery time than the larger 170L, IR sensor-equipped MCO 18M (Table 9.2). Sanyo improved the CO_2 recovery time of the MCO 5M 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 5M 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.

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.

Quality Control

Temperature

Incubator temperature drives the metabolic rate of the embryos in culture. Some authors believe to keep the incubation temperature 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 outperform conventional incubators without the Incuplates [19]. The volume of the culture medium, the volume of the oil overlay, and the use of a culture dish lid significantly affects 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 9.2). 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.

pH

Incubators should not be set for a specific CO_2 concentration, but rather with the goal to achieve a target pH such as 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 is critical and, sometimes, challenging. The pH probe should always be calibrated with buffers which bracket the target pH, and importantly, calibration should take place at the target temperature (37°C). In situ pH measurements of 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.

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

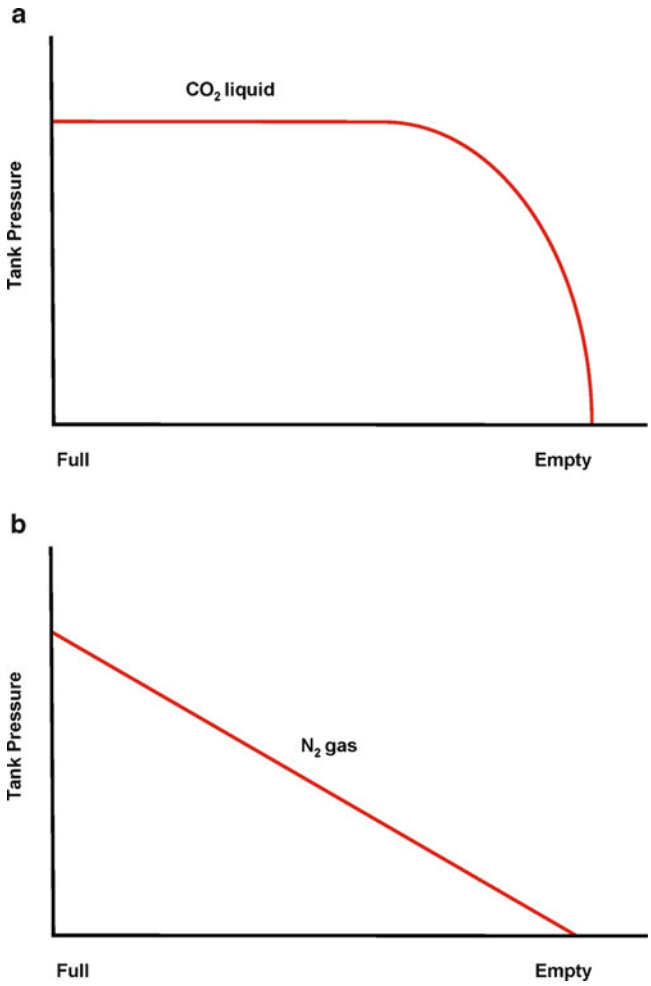

Fig. 9.3 (**a**) Because of the liquid state of the CO_2 supply and a constant pressure in the air space in the container above the liquid, one will not see a notable drop in manifold pressure until shortly before the cylinder runs empty. Liquid N_2 dewars will behave in the same manner. The manifold 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

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. 9.3). 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.

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, bench-top 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 introduce 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 at 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. 9.4). 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).

Siphoning pure N_2 gas off of 150 psi liquid nitrogen dewars instead appears to be a much more viable approach with

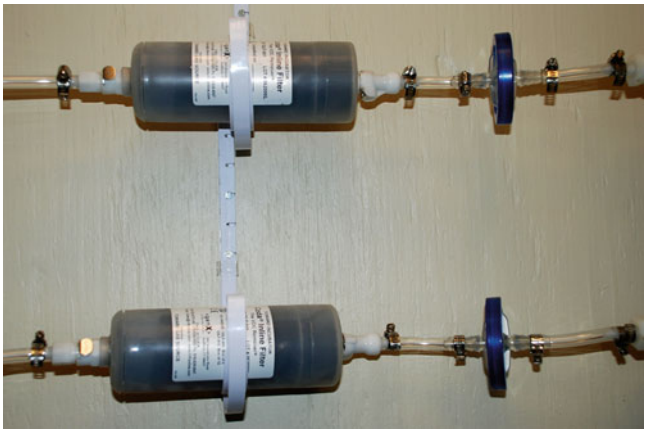

Fig. 9.4 All incubator supply gases should pass through a HEPA filter (particles) as well as a filter containing sufficient activated carbon/permanganate to remove gaseous contaminants

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}.

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.

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 shut down. 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) are 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 (option; 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 fumigates* 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.

Safety

Guidelines and requirements by most regulatory agencies with jurisdiction over IVF laboratories mandate that there should always be a back-up 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.

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 back-up 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.
- 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 back-up 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.

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IVF Workstations

10

Nicolas Prados and Alex C. Varghese

Abstract

Embryo development in vitro is dependent on many factors. It is mainly dependent on its own intrinsic potential (which depends on the couple characteristics and the response to the ovarian stimulation) and the conditions of culture. Human embryo culture, as other cell cultures, relies on external conditions for the well-being of the embryo. The main physical parameters are pH, temperature, light, and gas phase (carbon dioxide and oxygen concentrations) besides media composition. Incubators (as reviewed in Chap. 9) are designed to control these parameters during culture. Oocytes and embryos stay in them most of the time they spend in the laboratory. But everyday, for some time, they are taken outside the incubator to be observed or for changing media. It is common knowledge that the less time they spend outside the incubator, better embryo quality and gestational results we get. So in the last 20 years, IVF settings have evolved from cell culture standard laboratories with room temperature stages to specifically designed embryo-friendly settings specialized in embryo manipulation and culture with their own specifically designed equipment. Some of these improvements are reviewed in this chapter.

Keywords

IVF workstations • Laminar flow cabinet with a stereomicroscope • Microscope • Micromanipulation chambers • Closed workstations in the IVF laboratory

Embryo development in vitro is dependent on many factors. It is mainly dependent on its own intrinsic potential (which depends on the couple characteristics and the response to the ovarian stimulation) and the conditions of culture. Human embryo culture, as other cell cultures, relies on external conditions for the well-being of the embryo. The main physical parameters are pH, temperature, light, and gas phase (carbon dioxide and oxygen concentrations) besides media composition.

Incubators (as reviewed in Chap. 9) are designed to control these parameters during culture. Oocytes and embryos stay in them most of the time they spend in the laboratory. But everyday, for some time, they are taken outside the incubator to be observed or for changing media. It is common knowledge that the less time they spend outside the incubator, better embryo quality and gestational results we get. So in the last 20 years, IVF settings have evolved from cell culture standard laboratories with room temperature stages to specifically designed embryo-friendly settings specialized in embryo manipulation and culture with their own specifically designed equipment. Some of these improvements are reviewed in this chapter.

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Principles and Uses

IVF workstations are designed for the manipulation of human gamete and embryos. We can define manipulation as any procedure in which we disturb gametes or embryos from their culture in the incubator. Some of these procedures are more critical than others because of the amount of the environment change, the duration of it, or the sensitivity of the cell to the changes. The main points of manipulation would be:

- Sperm preparation
- Oocyte pickup
- Denudation
- ICSI
- Daily morphology checks (including fertilization)
- Media changes (usually, on first and third day of development)
- Embryo transfer

The four main parameters to take into account at a workstation are temperature, pH, oxygen, and light. They are very similar to those of the incubator as they are dealing with the same issues.

Temperature was the first parameter to be controlled in workstations. The cleavage rate of different mammal embryos is affected by exposure to room temperature [1, 2]. Human oocytes have also been shown to be sensitive to temperature drops as it disrupts the meiotic spindle [3]. Hyperthermia is also harmful, as it has been shown in rabbit embryos to induce heat shock gene expression and apoptosis [4]. Anyway, the empirical experience of years of IVF has taught us that one of the best ways to improve embryo morphology and implantation rates is to avoid temperature fluctuations during oocyte and embryo manipulation.

The pH of the culture is another important parameter which affects human embryos known for a long time [5]. Two strategies have been developed to maintain media pH: 25 mM bicarbonate/CO_2 buffer or non-CO_2 buffers. The first only relies in an atmosphere of 5–9% of CO_2 at sea level to maintain the pH between 7.2 and 7.4 (Henderson–Hasselbalch equation). Commercial media usually suggest adjusting the CO_2 concentration to achieve a pH around 7.3. The exact concentration depends on the medium composition and must be adjusted in each setting. Closed workstations must be used to maintain the CO_2 atmosphere. When gametes or embryos are manipulated with standard atmosphere, 20 mM HEPES or MOPS is used to preserve the pH. Media with one of these buffers are mandatory if working with oocytes or embryos for more than 5 min in open workstations. The study by Blake et al. [6] shows that there is a shift in pH (>7.45) at a rapid rate within 2 min of exposure of 50 mL droplets of culture media under oil kept in CO_2 incubator when exposed to atmospheric air. It has been noted that reequilibration to normal pH took around 35 min once the outgassed dishes were replaced in the CO_2 incubator. In contrast, the outgassed culture media (5 mL—kept in a dish) took 15 min to equilibrate once replaced in the incubator. These data warrant the exposure of droplets with embryos kept under oil and exposed to light for long time during zygote/embryo image acquisition or during ICSI in the bicarbonate buffered media.

Oxygen and light are more arguable parameters to be controlled. Culture of human embryos under low oxygen concentration has been proposed as advisable for a long time, especially for blastocyst culture [7, 8], and recent studies have shown a clear improvement in embryo quality and live births [9, 10]. Nevertheless, it is still not standard to culture human embryos in low oxygen concentrations, and thus, it is not very usual to control oxygen level in standard workstations in this moment.

Light is easily controlled, and, by a precautionary rule, most laboratories use filters on the microscopes and work in low ambient lights. Its uncontrolled exposure in the laboratory may be the least harmful of the four parameters, but it has still to be further researched and established [11].

There is a compromise between the parameters controlled, the cost, and the ergonomics of the system. New products are usually introduced as reasonable theoretical improvements without any testing or trials in human embryos. They do not in any case worsen what we already use everyday, but it is not clear the amount of benefit in live births, which is the main purpose. This makes difficult to evaluate if the investment in new equipment is worth the effort and cost.

A different issue in a new workstation is the ergonomics of the embryologist. The more complicated systems are sometimes more awkward or uncomfortable to use. The gain in embryo quality can also represent an increase in accident risks because of the limiting range of movement or the wearing of the embryologist. Care must be put in training with these new systems, as they sometimes require different attitudes or protocols of manipulation.

There have been three strategies in the improvement of workstations. Open workstations are traditional flow cabinets and microscope stages that have been adapted to maintain as long as possible the temperature of gametes and embryos. The next step has been closing them to maintain the gas phase, controlling the carbon dioxide concentration (and in some cases also the oxygen) and improving the temperature control. The last step has been to design culture systems where the embryologist can manipulate embryos without taking them outside the incubator, with special handholes or time-lapse video systems.

The Basics: Open Workstations

The traditional workstation unit usually consists of a vertical laminar flow cabinet with a stereomicroscope, a working incubator inside or nearby the cabinet, and an inverted microscope on an antivibration table. These must be placed in a way to minimize the distance and optimize the transport of embryos during their assessment or manipulation. Usually in larger settings, the workstation nearer to the operating theater does not have an inverted microscope as it is usually used only for oocyte pickup and embryo transfer. K-Systems introduced the actual standard of having a heated surface with the stereomicroscope embedded at the same level which has become an IVF workstation standard. The main controlled parameter is temperature. During manipulation, CO_2 is lost, so exposure to ambient atmosphere must be short, or HEPES buffer must be used. In IVF, where handling/manipulations of gametes or embryos are rapid, use of laminar flow hood may be more harmful than beneficial since the airflow may result in media desiccation, thereby osmolarity changes. The chances of temperature reductions are also common with open hoods as only the bottom side of the dish gets warmth. Moreover, with the cooled air-conditioner air in the IVF lab, the dish may not get enough heat transfer from the warm stage (37°C) of the hood where the stereo zoom microscope is usually placed to the periphery.

Microscopes

With a width of around a seventh of a millimeter, some magnification is needed in order to distinguish an embryo. Usually, a stereomicroscope (×10 to ×100 magnification) or a microscope (×50 to ×600 magnification) is used to visualize oocytes and embryos.

Stereomicroscopes

Stereomicroscopes, the work horse of the IVF laboratory, are used when low magnification is needed, usually cumulus isolation, washing and denuding, and embryo handling. It is not recommended for embryo morphology assessment (even for fertilization as micronuclei are not detected).

There is no need for a high magnification or an image of high quality. The magnification range of a routine stereo zoom microscope with 10× eye piece is 10–100×. With high magnification (over ×60), embryos are easily lost out of the field of view. This is more evident when embryologist's efficiency in fast activity is tested such as during vitrification process when embryos need to be shifted from drop to drop or to carrier devices in lightning speeds. So, for embryo handling, you do not need to have the most expensive stereomicroscope with the biggest zoom. Depending on the brand, there are different possibilities, but it is usually true that most add-ons (cameras and video recorders) only fit to the highest of the models.

Another issue is the optics. There is also no need for a high contrast objective (planar or flat-field lens) as the higher definition comes with a depth of field loss. Embryos move around the droplet when handling. Flat-field lenses give nice contrasted images, but they are very tiring to use as eyesight is more stressed when the embryo goes constantly out of focus.

The method of illumination is not menial. Oocytes and embryos have very little opacity. Usually, a transmitted (diascopic) diffuse reflected light is used for oocytes and embryos. The illuminator source reflects on a two-sided mirror which can be turned in order to get direct or diffuse illumination. Direct illumination (or a slight change in the angle of the reflector) can be useful to confirm the presence of mobile sperm cells in an insemination droplet. If the heat of the illuminator source can interact with the thermostability of the stage, a fiber optic pipe can be used. Sometimes the presence of two diffusors (one on the end of the fiber optic pipe and another in the reflector) can make a poor image. It is suggested then to use the direct illumination side of the reflector, as the other diffuser is enough to get a contrasted image.

Finally, embryos in droplets by the side of the plate are much more difficult to see properly as they are difficult to focus well enough because of the interference of the plastic with the optics. This is more distressing if air bubbles get trapped on the interfaces of media and plastic during denuding/changing zygotes from well to well. Air bubbles usually appear if one uses very narrow diameter denuding pipettes during cumulus removal 16–18 h after the insemination (fertilization check). Still many laboratories use mouth aspirator fitted with pipettes for denuding. During the aspiration of the clear culture media from one well, air bubbles may get trapped in the pipette, and when they move to the well with OCC and release the media, the air bubbles on its way carry away some oocytes to the media—oil interface or to the periphery. It is during the time of denuding process, a stereo zoom microscope's efficiency in embryology is evaluated properly and how less fatigue it causes to the embryologist.

Inverted Microscopes

Usually a standard cell culture inverted microscope is used for oocytes and embryo assessment with some kind of differential interference contrast developed for standard plasticware (as the original Hoffman modulation contrast). More specifics about inverted microscopes and their micromanipulation systems are reviewed in following chapters. Since the introduction of pronuclear morphology evaluation in embryo scoring, inverted microscopes play a major role other than for the routine micromanipulations in IVF. When a good

quality video camera along with image grabber/video grabber is attached to the inverted microscope, it gives the laboratory the options for recording the images. Sometimes, installation of low end camera or video grabber and monitor can give poor quality images or video of the procedures such as ICSI.

Laminar Airflow

In order to keep the aseptic conditions in the workstation, a laminar airflow cabinet is used. Horizontal flow cabinets are usually discouraged when manipulating human tissues as the operator is completely exposed to any liquid or aerosol produced. It is not recommended a class-II biosafety cabinet as the conditions inside would be nearly impossible to control and they would be too detrimental for the embryo. Usually, patients undergoing an IVF treatment are screened for infectious diseases, and universal safety rules are enough for handling gametes and embryos. If a laminar flow hood is used (especially if it horizontal flow), it should not be placed near the incubator as the airflow from the hood can cause dramatic changes in the osmolarity of the culture media kept in the incubator because of frequent openings.

Heated Stages

The standard workstation has a continuous heated surface with the stereomicroscope embedded, so there is no height change between the optic stage and the rest of the surface. Usually, under the heated surface, there are aluminum blocks heated by a water-based system or electric resistance system. The simplest water system can be just a closed circuit with a water bath with a pump, to the most complex of electronically controlled electric resistances where different areas of the surface can be independently adjusted.

On stand-alone microscopes (inverted or stereomicroscopes), a hot surface of metal or plastic is used. They are connected to a temperature controller unit. In the case of metal stages, objective warmers can be used in order to avoid the temperature drop in the center of the stage. Plastic stages have more homogeneous temperature but are more sensitive to scratches to the surface, which can worsen the final image. Linkam (United Kingdom) and Tokai Hit (Japan) are the most common suppliers of metal and plastic stages, respectively, though there are many others. Stand-alone stereomicroscopes should have a heated stage with large surface area so that 4-well dishes and 1–2 round Petri dishes can easily be accommodated while transferring embryos from one dish to another.

Gas Supply

In order to minimize the CO_2 loss (and the increase of pH), most workstations have a 5% CO_2 premix supply with glass hoods, a small incubator attached inside the workstation, or a standard incubator by the side. These are usually named *working incubators*, and they are used as a support and are used only during the handling and assessment. No embryos are left for culture in them.

Cleaning and Disinfection

One of the main differences of workstations and incubators is that there are nearly no manipulations inside an incubator (just taking in or out the embryos), so the risk of getting them dirty is lower than in a workstation.

There should be a daily cleaning routine at the end of the day (usually a three step (soap, water, 70% alcohol)) leaving everything for the next day. Any spillage should be immediately cleaned with dry tissue or wet with only water. If it must be cleaned more thoroughly, no oocytes or embryos should be manipulated until the laboratory is ventilated (depending on the general air system of the laboratory).

Starting Up and Turning Off

Traditionally airflow cabinets have two airflow speeds. The higher one is for working and disturbing the air inside the cabinet, and the slower one maintains the aseptic conditions if no movement or work is performed inside the cabinet. This speed should be set during the nonworking hours. If the cabinet is turned off, every morning a cleaning and disinfection routine must be set as the cabinet will be "contaminated" by the room air. Some of the newer models do not have this speed choice, and so they should not be turned off at all.

Theoretically, warm stages take less than half an hour to stabilize the temperature, but some peaks and drops can still happen. Some workstations can be scheduled to turn on at a determinate time. In other cases, a programmable mains plug can be used. In the long run, it is safer not to turn off the warm stages as the risk of not having a stage warm enough to work.

Independently of being disconnected or not everyday, all controlled parameters (temperature, CO_2, O_2) should be daily monitored the same as with the incubators.

Good Practice

Obviously aseptic techniques must be followed at all times. Although the oil layer proves to be a very good barrier for contamination, care must be always undertaken. The surface of the cabinet must be always clean and clear. It should not be a store place for the embryologist tools. Anything that is handheld should never be kept inside (pipettes, pencils, papers, etc.).

The time embryos are outside their incubator should be minimal. The objective of embryo assessment is to choose the best embryo of the cohort, not the improvement of their quality. So there is no advantage in transferring the best embryo if we have impaired it taking too long looking at it. It happens in several labs while checking pronucleus and embryo morphology at different objectives and image-acquiring process which makes the embryo dishes lying on the stage for longer periods.

As every setting is different (the equipment and the protocols), one of the things any embryologist should always do is to measure the temperature drop of every step depending on the time. For example, the culture plates are usually transported in a bigger plate (used as a tray): they should never be left on the heated surface on the tray because they will get cold quicker (there will be two layers of plastic and air between the stage and the droplet). Another issue that is usually underestimated is the use of the glass incubator hoods. They not only prevent (or at least slow down the rate) the outgassing of the plates but also prevent their cooling. They offer a good barrier against the airflow at room temperature produced by the cabinet. Any plate that is not being handled should always be inside a working incubator (inside the workstation or by its side) or under a glass hood (even if no gas is available).

The principal manufacturers of open chambers specifically designed for IVF are K-Systems (Denmark), MidAtlantic Diagnostics (USA), and IVFtech (Denmark), though there are many others.

Closed Workstations

Closed workstations control temperature and pH. There is no need for HEPES buffer. Usually, there are a warm heated stage and a closed circuit airflow. This airflow is also heated and sterile filtered (HEPA, VOCs, and sometimes UV treated also), but aseptic conditions are a little bit lost as the flow is turbulent inside the workstation.

There is also the matter of decontamination and cleaning in closed workstations. In open workstation, cleaning any spillage is straightforward. In closed workstations, the cleaning is more complicated. Though there are always standard HEPA and VOC filters, if methanol is used without stopping and opening the workstation, the fumes will be recirculating inside the workstation until completely trapped in the VOC filter. This may be an issue especially during oocyte pickups.

Portable Chambers or Isolettes

These workstations rely on standard incubators for the culture of the embryos, and they are mainly designed for oocyte pickups and handling of oocytes and embryos (not their assessment). It may be noted that, though heated stages can be quickly warmed up, the atmosphere temperature takes much longer. The air must warm all the walls and nonheated surfaces. In some cases, this can take more than an hour. Similarly CO_2 concentration is quickly achieved, but if oxygen is to be controlled, the time to displace the atmospheric oxygen is also quite long. It may be suggested that you should leave the temperature controller connected and the CO_2 control disconnected especially as the *isolettes* are not very air tight.

Mobile IVF-1 Chamber (MidAtlantic, Origio, USA)

This is maybe the closed workstation most used worldwide. It has the advantage of being portable and small. Temperature and CO_2 are controlled units. The ergonomics are good with the electronically variable height stand as any embryologist can adjust it quickly to his height. It is designed to work standing, which can be shocking at first but much more comfortable once used to it in the long run. One disadvantage is poor gas isolation, so it really consumes a lot of CO_2 if used as a standard incubator. CO_2 is also highly consumed during oocyte pickups as the hand ports are opened and closed continuously. Another disadvantage is a small and relatively complex working area with many angles and surfaces. It is not easy to clean thoroughly a spillage without disrupting the temperature and CO_2 conditions. There is no possibility of oxygen control. It can fit any stereomicroscope, but there is no possibility of an inverted microscope.

emCell-s (HD Scientific Supplies, Australia)

This system is very similar to the IVF-1 chamber. It can fit a stereomicroscope. Height can be mechanically adjusted, and there is also no oxygen control.

L323 IVF Incu Chamber (K-Systems, Denmark)

This chamber has the advantage that oxygen can also be controlled. Like the previous one, it is not completely sealed (the manufacturer calls it a semiclosed chamber), so gas consumption can be high especially when using a low oxygen atmosphere (nitrogen is pumped to replace the ambient oxygen). It has a better clear working area, as it follows the

standard K-Systems workstation design. It can provide up to 60% humidity. It is theoretically portable, though it is heavier and bigger than the previous ones. Height can be adjusted, and you can work seated or standing. Ideally, the workstation should be switched on hours prior to the actual ovum recovery time so as to get a warm stage and heated air in the chamber. The water in the pan also should be changed periodically to avoid any contamination and to obtain a good humidity in the chamber.

Egg Recovery Module (CellCura, Norway)

This is a fixed height nonmobile chamber. The priorities in this model are ergonomics and safety. Ergonomics are nice as everything is viewed on screens and foot pedals control zoom and focus of a proprietary stereomicroscope system that is covered under a plastic hood. There is a safety increase as a digital bar coding system is implemented. Only temperature is controlled inside. Infrared sensors record the temperature of culture medium in the dish, alerting the embryologist from potential damage to the oocytes and embryos.

Micromanipulation Chambers

These workstations are designed for inverted microscopes. They provide a controlled atmosphere during assessment and micromanipulation (ICSI and embryo biopsy).

emCell-i (HD Scientific Supplies, Australia)

This is very similar to their other model but with an inverted microscope with all the micromanipulation hardware. The benefit of performing ICSI in a mobile unit with a controlled atmosphere may be counterbalanced by the lack of a vibration-free system and a loss of ergonomics.

ICSI Module (CellCura, Norway)

There is also a micromanipulation model with a Zeiss inverted microscope system. Again only surface temperature and air quality (HEPA filtered) are controlled. Both CellCura chambers are modular and can be assembled in a continuous linear way. It has the best ergonomics, and safety is controlled by the bar code tracking system. There are no ocular lens, so ICSI is performed looking at the screen.

Incubator L (PeCon GmbH, Germany)

This is a transparent plastic hood that covers a standard inverted microscope providing a controlled temperature and CO_2 atmosphere. Designed initially for research and clinical genetics, it can be adapted to any standard inverted microscopes. It would be the easiest choice for performing micromanipulation in a controlled atmosphere as it can be adapted to any working unit.

Real Closed Systems

These are truly closed systems designed to specifically control the environment, so embryos can be manipulated and cultured in the same piece of equipment. Embryos are always kept in stable conditions. A major drawback is the cleaning and disinfection of the unit. As we are joining the concept of manipulation and culturing, the need for regular cleaning is higher especially after oocyte pickup.

Active IVF (Ruskinn, UK)

Russkin has great experience in anaerobic cell culture chambers, and they have designed this chamber for ART. It may have the most comprehensive parameters for the control of temperature, CO_2, O_2, and humidity and has an independent airlock. The gas consumption is very low, and the conditions are very stable. It has a Nikon stereomicroscope that can achieve high magnification. A standard inverted microscope can also be installed, though handling of embryos may become more troublesome. The image is viewed on a screen, and it has embryo image acquisition and database software. Height is not adjustable and hand ports are fixed.

VitroSafe (Vitro Safe Systems, UK)

This is probably the most comprehensive system as it is designed as a production line where the different cabinets are interlocked among themselves, so embryos are not exposed to the lab atmospheric conditions from pickup till transfer. All steps are performed inside the different units with stereomicroscopes and inverted microscopes (handling and micromanipulation). Height is fixed, and work is usually performed seated. The gloved apertures are very wide, and gloves can be changed without compromising the integrity of the sealed unit. There are eyepieces and cameras in microscopes. It is also a true closed system, so CO_2 and temperature can be controlled. Gas consumption is also low, and there are different ventilated hatches located within each isolator unit.

Embryo Assessment

Another way of circumventing the need of a complicated workstation for assessing embryos would be to use after ICSI a time-lapse video recording system. Thus, embryos are not taken outside the incubator, and no workstation is needed. These systems fall in fact into the incubators category, and the main ones we can find in the market would be the following.

PRIMO Vision (Cryo-Innovation, Hungary)

This would be the simplest concept: a special microwell-WOW dish is put on a compact, inverted digital microscope

connected to an external laptop computer. The system can be put in any standard incubator, so the culture conditions depend on the user. Microwell-WOW embryo culture dishes must be used: up to nine embryos are group cultured in small wells inside the same droplet cultured under oil. There would be one dish per PRIMO Vision unit and up to six cameras connected by firewire per computer. Embryos are not disturbed during the culture. A detailed discussion on the continuous embryo monitoring is dealt with in Chap. 48.

BioStation CT (Nikon, Japan)

This is a CO_2 incubator with a Nikon inverted microscope and a robotic arm that moves the dishes from a rack with 30 different positions (up to five 35-mm diameter plates in each position) to the microscope. It is designed for general cell culture, so any type of flask or dish can be used. Humidity and CO_2 are controlled, but not the oxygen level. It is not designed specifically for routine IVF, and care must be put in the way embryos are cultured to maintain focus and position.

Embryoscope (Unisense Fertilitech, Denmark)

This equipment is specifically designed for IVF. It can manage six special plates with 12 microwells in each plate (up to 96 embryos). It takes images at different focus planes of the embryos every 20 min with Leica optics with a Hoffman modulation contrast. Embryos are illuminated at 635-nm for a very short time for each image. CO_2 and O_2 are controlled, but not the humidity, so oil overlay must be always used. The incubator sends the images and information to a computer workstation where the embryos are assessed at any other time.

Conclusion

Most successful IVF centers around the world are using some kind of closed workstations. Though the open warm workstation originally designed by K-Systems is already standard, there is no consensus in which is the best approach for closed workstations as they all have advantages and disadvantages (Table 10.1).

Some of the closed workstations are very expensive, and the benefit has not been clearly stated. Some studies have been reported. In some closed systems, an improvement in embryo morphology and live births has been described [12, 13]. Improvement in embryo assessment and selection has been shown in the time-lapse systems [14]. There is no study comparing different workstations at the same time. It is obvious that having a better control of the environment, embryo quality, and success should improve, but much further work must be undergone before establishing a new standard.

Right now, a high-profile solution for a laboratory in order to have most of the advantages of each system would be (1) portable chambers for general handling (oocyte pickup, denuding, and transfer), (2) plastic hood incubator for the inverted microscope, and (3) time-lapse system for embryo culture and assessment.

In light of several reports of genetic or epigenetic defects in the children borne by assisted reproductive technologies, the embryologist along with the hospital management should look critically at various instruments and embryo handling systems and select the ones which mimic the in vivo environments. By doing so, we can ensure less exposure to outside deviations and thereby less perturbations to molecular integrity of gametes and embryos.

Table 10.1 Characteristics' summary of workstations for handling of embryos

		Isolettes (semiclosed systems)			Closed systems	
Workstations	Open	IVF-1/emCell	L323	CellCura	Active (IVF)	VitroSafe
Airflow	Laminar	Turbulent	Turbulent	Laminar	Laminar	Laminar
Air quality	HEPA	HEPA	HEPA, VOC, UV	HEPA	HEPA, VOC	HEPA, VOC
Temperature control	Only on surface and optic stage	Entire chamber	Entire chamber	Only on surface and optic stage	Entire chamber	Entire chamber
CO_2 control	–	+	+	–	+	+
O_2 control	–	–	+	–	+	–
Humidity control	–	±	±	–	+	–
Airtight	–	(+)	(+)	–	+++	++
Cleaning	+++	+ Disrupting	++ Disrupting	+++	+ Disrupting	+ Disrupting
Embryo viewing	Direct	Direct	Direct	Monitor	Monitor	Direct
Height	Fixed	Adjustable	Adjustable	Fixed	Fixed	Fixed
Other strong points		Mobile	Working space	Safety, tracking, ergonomics	Programmableatmosphere control	Comprehensive modularity

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Culture Media in IVF: Decisions for the Laboratory

11

Jason E. Swain and Thomas B. Pool

Abstract

Appreciation of the evolution of IVF culture media, highlighting salient discoveries that have led to the tremendous improvement over just a few short years ago, is instrumental in gaining insight into the complexities of gamete and embryo function. In turn, this knowledge brings understanding to the rationale behind current laboratory practices and aids in the ability to make informed decisions in regard to culture methods. Furthermore, discussion of impact of culture media on homeostatic regulation of gametes and embryos, focusing on key decisions made within the laboratory such as media type, macromolecule selection, and pH, further highlights their delicate nature, the need to minimize stressors, and ultimately provides insight into areas where future improvement can be made as we continue to strive for improvement in IVF success rates.

Keywords

Embryo • Oocyte • Amino acids • pH • Glucose • Culture media in IVF • IVF culture media

Substantial research has been undertaken with the intent of 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]. One would think that the most appropriate course of action would have been to adopt the media and culture principles being developed for animal embryos, yet clinical IVF underwent an expansive phase in the early to mid-1980s by instead using the expedient approach of borrowing simple salt solutions and complex media formulations developed for somatic cell culture [3]. Although the field is now benefiting through the clinical use of media developed systematically for mouse embryos in the laboratory of Professor John Biggers, it was through the work of pioneers like Quinn and Menezo that media designed specifically for human embryo culture in vitro initially became available [4, 5]. 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 [6, 7]. 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, macromolecule supplement, and pH, are still directly controlled within the IVF laboratory and require special attention. Review of these topics is necessary to optimize laboratory efficiency and ultimately IVF outcomes.

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Laboratory Choices and Culture Media

Mono vs. Sequential Culture

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. Though there is a clear consensus on the advantages of commercial media production, an ongoing debate exists with regard to the selection of optimal IVF culture media. In the last 10–15 years, there has been 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, then 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 (Fig. 11.1). 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 [8]. 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.

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 [9]. The approach of replenishing media at 2–3-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 [10–12].

Extensive data on the release or depletion of nutrients by both mouse and human embryos led Leese [13] 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 such as heat shock and nucleic acid damage [13, 14]. The extent to which "noisy metabolism," induced by stresses from the in vitro environment, influence 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

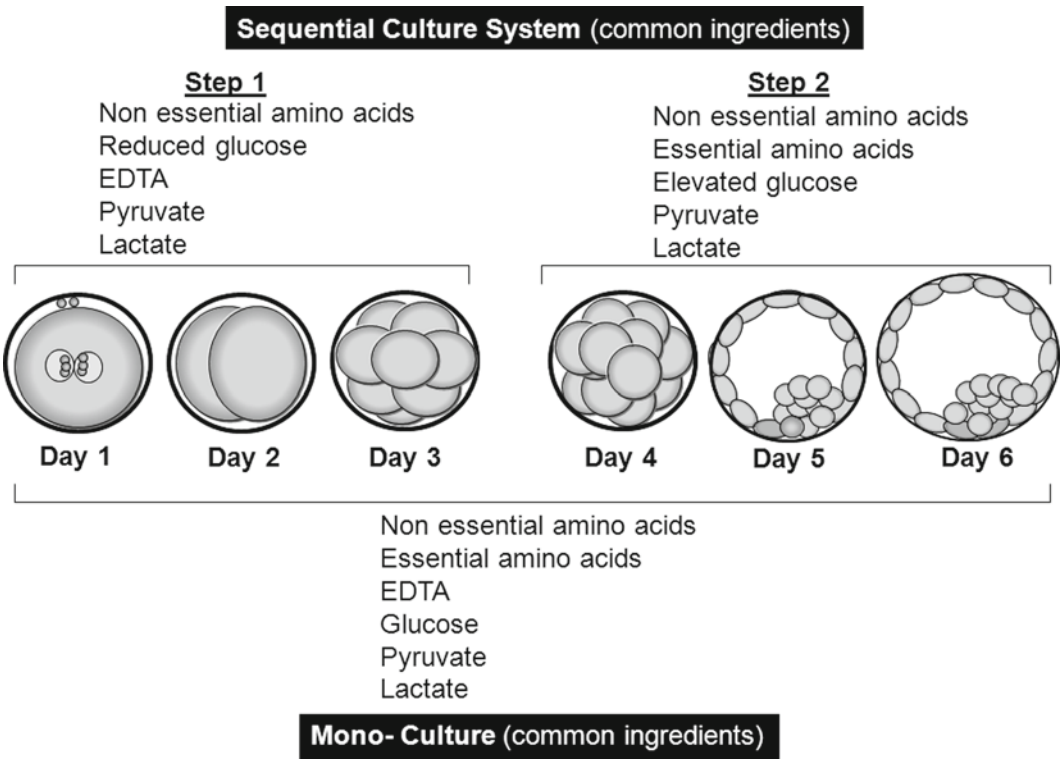

Fig. 11.1 Key energy substrate components of embryo culture media

development, their efficiency needs to be evaluated in the context of their use within a particular laboratory, in conjunction with the variety of other processes that make up the complete culture system [15]. 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. 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.

Energy Substrates

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 [16–19]. Indeed, sequential systems utilize reduced levels of glucose (0–0.5 mM) in the first step of their system and also often include EDTA, which further suppresses glycolysis [20]. Furthermore, culture media devoid of glucose support high rates of human embryo development and pregnancy during the first 3 days of culture [21–24]. 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 mammalian embryos primarily utilizing glucose during compaction and blastocyst development. Therefore, the second media employed in sequential systems raise the concentration of glucose (>2.0 mM).

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 which 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 [25]. Furthermore, pyruvate was discovered to be imperative for cleavage of one-cell mouse zygotes and is the primary energy substrate for cleavage stage embryos [26–28], including human, where it is used throughout development to the blastocyst stage [17, 29, 30]. Importantly, low levels of lactate can affect pyruvate metabolism [31, 32] and can also acidify internal pH [33, 34]. It has been suggested that only the metabolizable L-lactate isomer be included in culture media formations to avoid excess suppression of internal pH that may exist with use of D-/L-isomers [35].

Amino Acids

Amino acids can not only serve as energy sources to developing embryos but also serve to maintain intracellular homeostasis by acting as zwitterions to buffer pH, antioxidants, and as organic osmolytes to control cell volume [36]. 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 [37]. 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 [38]. 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 [39–41]. Indeed, even brief exposure of mouse zygotes to media lacking amino acids can impair development [42]. 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 resulting offspring [43–47]. Though debated, of particular interest is glutamine, which is the most labile of the amino acids [48]. Fortunately, dipeptide forms of glutamine, such as alanyl-glutamine [46] or glycyl-glutamine [49, 50], are stable at 37°C and help alleviate this concern. Therefore, the use of media utilizing these stabile forms of glutamine is recommended.

Osmolality

An important concept to be aware of 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 [51]). Osmolality of commercial culture media varies (Table 11.1). 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 that provide

Table 11.1 Osmolality ranges of various commercial media utilized in IVF

Media	Osmolality range (MOsM)
Irvine	
P1	282–298
ECM	282–295
Single-step (SSM)	280–295
Multiblast	281–291
HTF	272–288
SAGE	
Fert media	265 ± 8
Cleavage media	265 ± 8
Blastocyst media	265 ± 8
IVM	265 ± 8
Vitrolife	
GIVF	279 ± 5
G1.5	261 ± 5
G2.5	260 ± 5
Life global	
Global	260–270 (265)
Global Fert	280–292 (285)
Blastocyst	260–270 (265)
HTF	280–292 (285)
HTFxtra	280–292 (285)
Medicult	
Universal IVF	272–288
ISM1	272–288
ISM2	272–288
EmbryoAssist	272–288
BlastAssist	272–288
Cook	
Sydney IVF cleavage	285–295
Sydney IVF blastocyst	285–295
Sydney IVF Fert	285–295

protection against high osmolality [52–54] and are thus recommended in all media to aid in this regulation [51]. Special concern exists in changing from one media to another, such as between handling media and culture media with differing ion composition. 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. Use of larger volumes of media helps combat resulting osmolality rise, and oil overlay and use of humidified incubators are also recommended to prevent dramatic increases in osmolality.

Macromolecules

Macromolecules are an especially important consideration in IVF culture medium since their source, composition, and concentration are still largely decided within individual laboratories (Table 11.2). Therefore, as embryologists, it is our responsibility to do our due diligence and familiarize ourselves with macromolecules and their effects to help make informed decisions as to their use.

Table 11.2 Various macromolecules commercially available for inclusion in IVF culture media

Macromolecule	References
Human serum albumin (HSA)	
Recombinant albumin	[51, 52]
SSS (HSA + α (alpha) and β (beta) globulins)	[53, 54]
Dextran (DSS)	[3]
Hyaluronan	[55–57]

As IVF culture media advanced, the field moved toward use of a fully defined media, and the use of serum was abandoned. However, protein and other macromolecules still prove beneficial by improving embryo development, increasing cryotolerance, and also acting as surfactants and increasing colloidal osmotic pressure [55]. A common protein source utilized for in vitro culture is serum albumin, although lot-to-lot variation can be a concern [56], as well as presence of various contaminants. Therefore, recombinant albumin is also used and been found to be as effective or superior to albumin in animal models [57, 58]. 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 [59] and live birth outcomes [60] and may be concentration dependent [61]. Similar products are now widely available from various commercial media companies. Additionally, macromolecules such as hyaluronan are advantageous when included in culture media [62–64]. 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 [59]. 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 [3]. This combination supported embryonic developmental rates equivalent to albumin with alpha and beta globulins and produced clinical pregnancy [3, 59]. This work led to the trial of hyaluronan as a medium supplement by others. 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 the reasoning for this is unclear as the protein 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 use of increased protein concentrations. Macromolecules also impact cryosurvival (see review [65]). Another important factor to consider and monitor when utilizing macromolecules is their affect on media pH.

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 we routinely measure this parameter indirectly during daily laboratory quality control assessments of the atmospheric 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 on a day-to-day basis directly controls this aspect of culture media. Thus, greater attention to detail is given to pH in this chapter.

The pHe of culture media is simply a measure of hydrogen ion concentration, which is the result of the equilibrium between two primary components: the dissolved bicarbonate supplied in the media and the amount of 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, installing inner incubator doors, minimizing dish time out of the incubator, and using 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. The reasoning behind the need to stabilize pHe is evident from the work of Dale et al. [66]. Though mammalian embryos have various mechanisms to regulate their intracellular pH (pHi), including the Na^+/H^+ antiporter and HCO_3^-/Cl^- transporter, excursions in pHe are readily converted to variances in intracellular pH (pHi), at least initially. Additionally, oocytes have a greatly reduced ability to regulate pHi [67–69], thus reinforcing the technical need to establish a target pHe and conform to it during procedures that allow pHe to drift.

pH and Cellular Function

The need to establish a target pHe is clear when one considers pH controls several intracellular processes that can impact embryo development. Raising (~7.4) or lowering (~6.8) pHi in mouse embryos for only 3 h disrupts localization of mitochondrial and actin microfilaments compared to controls (~7.2) [70]. Even minor rises in pHi can also dramatically impact embryo metabolism through regulation of various enzymes, such as phosphofructokinase (PFK). Raising pHi ~0.1–0.15 units significantly increased embryo glycolysis and lowered oxidative metabolism [33, 71], which can dramatically impact developmental competence. Importantly, other common practices in IVF, such as vitrification, reduce the ability of these already sensitive cells to regulate pHi for about 6 h [71]. Finally, more recent evidence suggests that pHe 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 [72].

Special concern for pHe exists with respect to denuded mature oocytes. These sensitive cells lack or have diminished ability to regulate pHi and are therefore heavily dependent upon pHe. Thus, in procedures such as ICSI, where the protective cumulus cells are purposefully removed, cells are created that are extremely susceptible to perturbations in pHe until several hours after fertilization occurs [73–76]. Little work has been done examining the effects of pHe on the mature oocyte. This should be concerning given the prevalence of oocyte-derived aneuploidy and the potential impact pH has on the meiotic spindle. pH is known to affect embryo actin cytoskeletal elements [70], and the oocyte cytoskeleton is responsible for positioning of the meiotic spindle [77, 78]. It is known that alkaline pH affects microtubule assembly/disassembly in bovine brain cells [79], and similar actions may be occurring with the meiotic spindle within the oocyte. Additionally, because pH can affect embryo mitochondrial localization [70], the same may hold true for oocytes. This is concerning because the distribution of oocyte mitochondria is correlated with developmental competence [80, 81]. Oocyte metabolism is also likely affected by pH, which, in turn, has been correlated with maturational status and developmental competence [82, 83]. All these factors indicate a strict regulation of pHe and avoidance of periodic deviations when dealing with oocytes.

Optimal pHe

The ability of embryos to regulate pHi is evidenced by various studies that show embryos can to develop over a pHe range of ~7.0–7.4 without any discernable effect on pHi or development [84, 85], while excursions of pHe outside this range have deleterious effects on embryo developmental competence [75, 86–89]. 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 pHi of around 7.1 likely stresses the embryo, as more resources are required to maintain the proper pHi. Conventional wisdom tells us that pHe of culture media should be slightly higher than pHi 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 no "optimum" pHe for oocytes and embryos, as this will vary from medium to medium based on specific ingredients. The amount of monocarboxylic acids, such as lactate and pyruvate, in culture media can lower pHi [33, 34]. Additionally, amino acids, particularly glycine, taurine, and glutamine, act as zwitterions and help in buffering pHi [34]. Though anecdotal observations from various laboratories suggest that perhaps culturing cleavage-stage embryos closer to pHe 7.2 may give better embryo development, the influence of media composition cannot be overlooked. Embryos grown in media with different amounts of monocarboxylic acids may have different pHi, though the pHe may be the same. 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 [90–92]. 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 7.4. At the moment, there is no "ideal" pHe at which to culture embryos, and this is reflected in the wide ranges of acceptable pHe given by various commercial media companies (Table 11.3). Furthermore, despite the growing trend, it remains unknown whether early cleavage stage embryos prefer a slightly lower pHe than later stages of embryo development. However, it is known that later stages of embryos with tight junctions, like the morula and blastocysts, can regulate their pHi more rigorously than early cleavage stage embryos and can thus tolerate a wider range of pHe [33]. Interestingly, there are data to suggest that a slightly more alkaline pHe may benefit fertilization. Dale et al. [66] found higher rates of sperm binding to empty zona pellucida 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).

Table 11.3 Recommended pH ranges for various commercial IVF culture media

Media	Recommended pH range
Irvine	
P1	7.27–7.32
ECM	7.2–7.25
Single-step (SSM)	7.8–7.32
Multiblast	7.3–7.4
HTF	7.2–7.3
SAGE	
Fert media	7.3 ± 0.1
Cleavage media	7.2 ± 0.1
Blastocyst media	7.3 ± 01
IVM	7.2 ± 0.1
Vitrolife	
G5 Series	7.27 ± 0.07
Life global	
Global	7.2–7.4[a]
Global Fert	7.2–7.4[a]
Blastocyst	7.2–7.4[a]
HTF	7.2–7.4[a]
HTFxtra	7.2–7.4[a]
Medicult	
Universal IVF	7.3–7.4
ISM1	7.2–7.3
ISM2	7.35–7.45
EmbryoAssist	7.2–7.3
BlastAssist	7.35–7.45
Cook	
Sydney IVF cleavage	7.3–7.5
Sydney IVF blastocyst	7.3–7.5
Sydney IVF Fert	7.3–7.5

[a]Company recommends optimal of 7.3 and suggests user adjusts CO_2 readings to reach this after taking into account protein supplementation

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 cannot be stressed enough. As reviewed in this chapter, the introduction of more sophisticated culture medium formulations for use within the incubator has dramatically improved our ability to produce better embryos in vitro. Altering energy substrate concentrations, use of either sequential culture or contemporary monoculture, incorporation of amino acids, and introduction of complex protein sources have all proved beneficial. However, the composition of media used for manipulations outside the laboratory incubator has been largely overlooked. 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 [93, 94]. 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 (PBS) solutions, 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

Table 11.4 Common zwitterionic (Good's) buffers and their p*K*a values (optimal buffering) in relation to temperature

Buffer	pH range	p*K*a 25°C	p*K*a 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

phosphate, may compromise gamete and embryo metabolic activity, disrupt organelle distribution, and interfere with intracellular ionic homeostasis, including intracellular pH [95–97]. Indeed, even brief exposure to PBS as a handling media has been shown to compromise hamster and rabbit embryo development [98, 99] and results in aberrant gene expression in bovine embryos when compared to other buffers [100]. 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 [101–103] (Table 11.4). However, different cell types display varying sensitivity to individual zwitterionic buffers [103]. 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 p*K*a values, an indication of their optimal buffering capacity.

Historically, HEPES at ~21 mM has been a standard for IVF handling media. More recently, at least two commercial companies also now include MOPS in their IVF handling media. 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 [104, 105]. 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 pharmacological 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 [106, 107]. This may have also been a factor in the observations of Butler et al. [108]. Though no data were presented, authors commented that inclusion of HEPES in the medium resulted in increased oocyte degeneration; however, the medium contained riboflavin. Several studies actually indicate that HEPES is able to support oocyte maturation [109–111], fertilization [111–115], and embryo culture [104, 111, 114, 116–119]. Those studies indicating lower fertilization rates in the presence of HEPES are likely due to the simultaneous reduction in bicarbonate concentrations [115]. Embryo development is supported in the presence of HEPES when bicarbonate is present, but not when bicarbonate is absent [116]. 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 [120–122] 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 [94, 123]. 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. 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 p*K*a of synthetic organic buffers, as well as the actual pH of the media, changes as temperature changes [94]. 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 p*K*a of 7.2 is the closest of the 20 zwitterionic buffers to the pHi of embryos of 7.12 [124]. Thus, MOPS would seemingly offer the best pH buffering of available options. However, the p*K*a of 7.2 for MOPS is at 25°C. Many laboratories warm their handling media to around 37°C, a temperature at which the p*K*a 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.

Future Directions

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

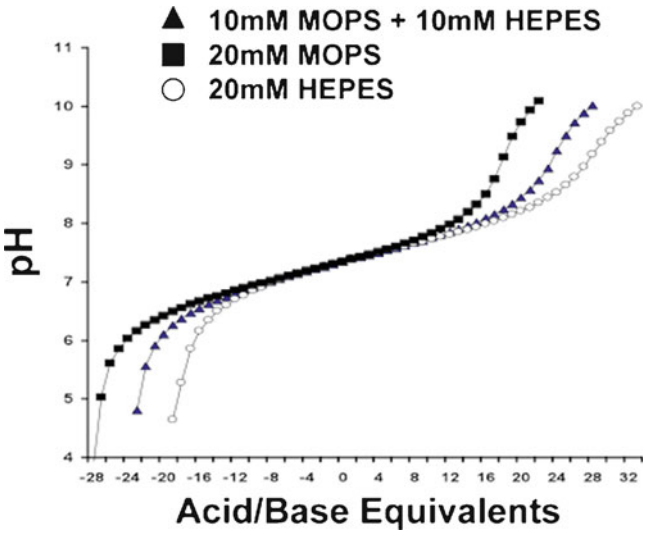

Fig. 11.2 Combining buffers allows for adjustment of p*K*a at specific temperatures

into a single solution [125]. Combining buffers allows for selection of an exact p*K*a value at a given temperature, which may be more appropriate and offer better buffering than a single buffer (Fig. 11.2). 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 20 mM of buffer. Indeed, this approach is a valid substitute for current monobuffered handling media. Swain and Pool [94] verified that bi- and tri-buffered media with combinations of zwitterionic buffers support mouse embryo development. Preliminary data suggest that these buffers may be beneficial for ICSI over monobuffered media [94]. With further testing and verification, additional buffers 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.

Media Additives-Antioxidants

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 [49, 50, 126]. A similar approach could be explored with other ingredients, such as pyruvate. In addition to its role as a metabolic substrate, pyruvate also acts as a powerful antioxidant [127] and can protect embryos from harmful reactive oxygen species (ROS) [128–130]. However, sodium pyruvate, the form of pyruvate added to all current embryo culture media formulations, is labile in solution [131]. By-products of pyruvate breakdown interfere with key steps in the TCA cycle, thereby compromising metabolic activity [131, 132], 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 [132]. A potential improvement over current use of sodium pyruvate in embryo culture media entails 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 [134–144]. Interestingly, both ethyl and methyl pyruvate were superior to sodium pyruvate in supporting mouse embryo development when pyruvate was the sole energy source [145]. Thus, these esterified forms of pyruvate may lend added benefit due to increased stability in solution or increased membrane permeability, factors which could have an impact on both or embryo metabolism or ability to scavenge ROS. Indeed, supplementation of IVF media with other antioxidants could provide useful in providing added protection and reduced intracellular stress to developing cells [146, 147].

Progressive Media and Microfluidics

Perhaps the most intriguing possibility for the improvement of IVF culture media lies in its use in conjunction with another technological development known as microfluidics. Through the use of automated microfluidic devices, one can 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 [148], by providing a customized growth environment suited to the specific needs of particular embryos, rather than expecting all embryos to adapt to a predetermined environment.

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Part III

In Vitro Fertilization

Oocyte Denuding

12

Roberta Maggiulli, Filippo Ubaldi, and Laura Rienzi

Abstract

The term *oocyte denuding* refers to the removal of the somatic cell layers that surround the oocytes. During the preovulatory growth, within the follicle, the oocyte is surrounded by two different cell layers: *granulosa and thecal cells* that sustain oocyte nutrition and maturation providing essential metabolites, hormones, and growth factors. However, the oocentric perspective of folliculogenesis highlights the central role of the oocyte itself in directing its own development as well as the follicle differentiation. In this regard, the oocyte is deemed capable of modulating the follicular environment in order to guarantee correct preantral, antral, and preovulatory development.

Keywords

Oocyte denuding • Cumulus–corona–oocyte complex harvesting • Oocyte cytoplasmic evaluation • Extracytoplasmic structure evaluation • Hyaluronidase or coronase in oocyte denuding

Overview of the Technique

The term *oocyte denuding* refers to the removal of the somatic cell layers that surround the oocytes. During the preovulatory growth, within the follicle, the oocyte is surrounded by two different cell layers: *granulosa and thecal cells* that sustain oocyte nutrition and maturation providing essential metabolites, hormones, and growth factors [1–4]. However, the oocentric perspective of folliculogenesis highlights the central role of the oocyte itself in directing its own development as well as the follicle differentiation [5–9]. In this regard, the oocyte is deemed capable of modulating the follicular environment in order to guarantee correct preantral, antral, and preovulatory development.

During folliculogenesis, granulosa cells differentiate in two different specialized subpopulations: the cumulus oophorus, 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 oocytes 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, a considerable active secretion of hyaluronic acid occurs, causing further expansion of cumulus–corona mass that appears finally as an expanded and mucified layer. The hyaluronic acid is a glycosaminoglycan that interposes among cells and separates them, conferring to the cumulus–corona mass a fluffy appearance. Whereas in natural cycles the stage of oocyte nuclear maturation is coupled to an increased expansion and mucification of the cumulus layer [12], in stimulated cycles, it may be observed as asynchrony of these two processes [13].

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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].

When oocyte denuding is thus performed, the oocyte is exposed for a variable amount of time to culture conditions without the protection of these layers and prior to its activation induced by the sperm entry at fertilization. The effect of this is unknown and still unexplored.

Ovum Pickup and Cumulus–Corona–Oocyte Complex Harvesting

In stimulated cycles, oocyte retrieval is usually performed at 36 h after hCG administration by ultrasound-guided transvaginal aspiration. The harvesting is carried out using an aspiration unit composed by 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 pressure are available commercially. Since during the collection, oocytes are exposed to an increasing pressure that may affect their integrity, a maximum pressure of about 120 mmHg is recommended to avoid the risk to damage the oocytes [17]. Moreover, to minimize changes in temperature, the collection tubes should be kept in a tube warmer before being connected to the collection system.

Once the ovaries are visualized, ovarian follicles are aspirated in a systematic fashion. Immediately after the collection, the adjoining laboratory examines the follicular fluid. Cumulus–corona–oocyte complexes (CCOC) are identified in sterile plastic dishes, rinsed, transferred in preequilibrated 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 to the oocyte competence.

Indications

The evaluation of oocyte morphology prior to standard IVF is tricky because of the presence of the somatic cells. 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, 18–20].

With the introduction of assisted-fertilization techniques such as PZD (partial zona dissection), SUZI (subzonal insemination), and finally ICSI (intracytoplasmic sperm injection) [21], the removal of cumulus cells became a crucial step before fertilization since it allowed an unequivocal evaluation of oocyte nuclear maturity and easier handling of the oocytes during the micromanipulation procedure. 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 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 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 [22].

Equipment and Procedure

The cumulus and corona cells are removed prior to any micromanipulation procedure by using a combined enzymatic and mechanical treatment [23]. 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, normally covered by oil, on a warming plate.

A limited number of COCCs are normally placed into the first well/droplet containing the enzyme to disperse the cumulus cells (Fig. 12.1a). To enhance enzymatic removal of

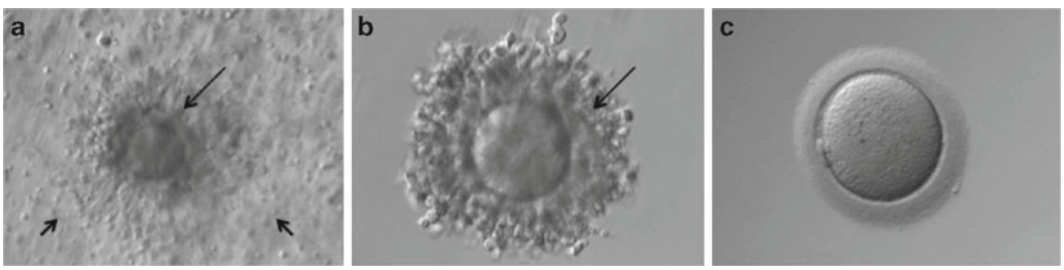

Fig. 12.1 Oocyte denudation steps. (**a**) Oocyte surrounded by corona radiata (*short arrow*) and cumulus cells (*long arrow*); (**b**) oocyte after the enzymatic-induced dispersion of cumulus cells; (**c**) MII denuded oocyte

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. 12.1b), oocytes are moved to the second well/droplet containing clean buffered medium, taking care to carry over a minimum amount of enzyme. Then, further denudation is carried out by glass or plastic denuding pipettes with decreasing inner diameters (170–130 µm) to remove the corona cells (Fig. 12.1c). An accurate washing of denuded oocytes is then required to wash out completely the enzyme.

It has been proposed that a two-step denuding procedure involving an incubation period after oocytes exposure to the enzyme and before the mechanical step [24] 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 preequilibrated culture medium and place 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 150-µm inner diameter stripper. After denudation, the oocytes can be examined under an inverted microscope to assess integrity and the stage of maturation.

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. [25] postulated that preincubation of cumulus–corona–oocyte complexes before conventional insemination was beneficial for the accomplishment of complete oocyte maturation and assured high rates of fertilization and embryo development in vitro.

However, no differences between the effect of early (1–2 h after retrieval) and late (5–6 h after retrieval) denudation and injection on early embryo development was reported by Van de Velde [26]. The authors assumed that the stimulation protocol involved in the study allowed a well-synchronized nuclear–cytoplasmic maturation hiding the potential contribution of 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 [27]. In agreement with Ho and coauthors [28], 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. [29] who showed that oocytes retained sufficient potential for fertilization between 1 and 9 h after oocyte collection. On the other hand, they observed that extended preincubation time might be responsible of reduced embryo quality.

Oocyte Evaluation

Cumulus–Corona–Oocyte Complex 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 [19, 30–36]. Early studies from Rattanachaiyanont et al. [19], 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 CCOC scoring related to fertilization and pregnancy rates [34] as well as to blastocyst quality and development [35]. Recently, Lin and colleagues [35] proposed a grading system of COCCs based on the morphology of the oocyte cytoplasm, cumulus mass, corona cells, and membrana granulosa cells, for oocytes prior to insemination by conventional IVF. Five grades (*mature group*, *approximately mature, immature*, *postmature, and atretic*) were previously described and reported in Table 12.1. The authors reported higher fertilization rates for the oocytes belonging to the mature group compared to those belonging to the other groups. Moreover, the immature group was characterized by a higher incidence of poor morphology day 3 embryos as compared to the mature group.

It has also been suggested that the presence of COCC anomalies such as blood clots or amorphous clumps may be an index of a suboptimal follicular maturation [37, 38] and may impair embryo ability to develop to blastocyst stage. When these oocytes are denuded of their cumulus cells, a significant alteration in cytoplasmic texture has been highlighted [39] that may be related to the reduced fertilization rate obtained when those oocytes are used for insemination [38]. Variation in temperature and pH as well as reactive oxygen species (ROS) induced by the presence of blood clots is pointed to be at the origin of the compromised competence of those oocytes [32].

Although, cumulus–corona mass observation is not sufficient to evaluate oocyte maturity and competence, it is reasonable to hypothesize that ooplasm development is influenced by the action of these cells. In accordance with this hypothesis, it has been suggested that cumulus–corona cells play an important role in the in vitro maturation of oocytes that were immature at the time of retrieval [40, 41]. Therefore, a careful observation of CCOC morphology may

Table 12.1 Cumulus–corona–oocyte complex evaluation

Groups	CCOC morphology
Mature	Expanded cumulus Radiant corona Distinct zona pellucida, clear ooplasm Expanded well-aggregated membrana granulosa cells
Approximately mature	Expanded cumulus mass Slightly compact corona radiata Expanded well-aggregated membrana granulosa cells
Immature	Dense compact cumulus if present Adherent compact layer of corona cells Ooplasm if visible with the presence of germinal vesicle Compact and nonaggregated membrane granulosa cells
Postmature	Expanded cumulus with clumps Radiant corona radiata, yet often clumped, irregular, or incomplete Visible zona, slightly granular or dark ooplasm Small and relatively nonaggregated membrana granulosa cells
Atretic	Rarely with associated cumulus mass Clumped and very irregular corona radiata if present Visible zona, dark and frequently misshapen ooplasm Membrana granulosa cells with very small clumps of cells

Adapted from Lin et al. [35], with permission

be a useful tool for oocyte selection in those circumstances where no direct evaluation of the oocyte is possible. In addition, in our laboratory, where a limited number of oocytes can be inseminated accordingly to the Italian law, we evaluate CCOC prior to denudation for ICSI. In fact, as suggested by Canipari et al. [42], we assume that there is a higher probability to obtain a better quality mature oocyte in a normally expanded cumulus than in a nonexpanded one.

Oocyte Cytoplasmic and Extracytoplasmic Evaluation

It is generally recognized that a "normal" human metaphase II oocyte, at light microscopy level, 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 [36, 43–48].

However, the majority of the oocytes retrieved after ovarian hyperstimulation exhibit one or more variations of the described "ideal" morphological criteria [36, 44–50]. This is true also for oocytes obtained from proven fertile donors [51]. The actual negative impact of the different oocytes' cytoplasmic and extracytoplasmic "abnormalities" is unclear.

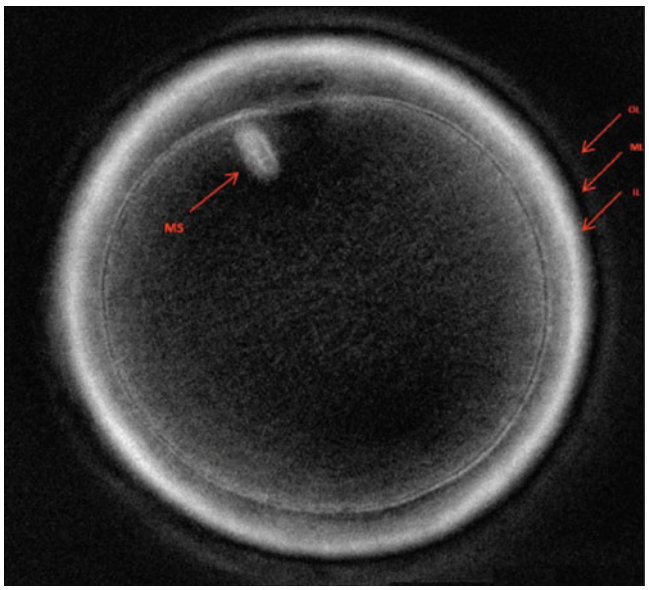

Fig. 12.2 Metaphase II oocyte observed by polarized light microscopy. *MS* meiotic spindle; *IL* zona pellucida inner layer; *ML* zona pellucida middle layer; *OU* zona pellucida outer layer

Conflicting data have been, in fact, published in the literature concerning oocyte morphology and ICSI outcome [36, 48].

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; the oocyte has still to enter the second meiotic division where a second arrest occurs at metaphase stage (MII). 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 nondisjunction of chromatids, chromosome scattering, and to the formation of aneuploid embryos [54, 57, 58].

The presence of the 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 assessed (Fig. 12.2). For this purpose, novel polarized light microscopy systems together with their image processing software (Spindle View Pol-Scope system, CRI, Woburn, MA, USA; OCTAX polarAIDE, MTG,

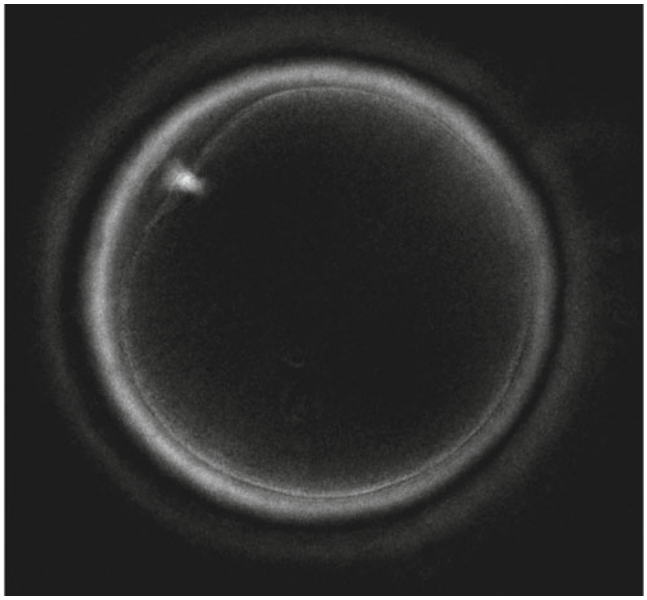

Fig. 12.3 Telophase I oocyte with the meiotic spindle interposed between the polar body and the cytoplasm

Table 12.2 Parameters that can be considered during MS visualization with polarized light microscopy

Presence/absence of MS, which may be due to
Laboratory factors
Temperature instability
Chemical and physical stress during handling and culturing
Correct oocyte rotation during visualization
Oocyte maturation, which is related to
Time elapsed from hCG administration
Interval between first and second meiotic divisions
Maternal factors (i.e., age)
Position and appearance of MS
Allow identification of immature oocytes (late telophase I)
Allow assessment of IPB/MS relative position, which affects oocyte fertilization rate and the orientation of the I cleavage plane
Allow the correct orientation of MS during ICSI
Quantitative data
MS pole to pole length, which is related to oocyte competence
MS mean retardance, which is a measure of MS polimerization

From Casciani and Rienzi [76], with permission

Germany) have been recently introduced in IVF laboratories allowing a noninvasive observation of the MS in living oocytes [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 composing 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 microtubules density [60, 63, 64].

The Pol-Scope allows 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 (Fig. 12.3). 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. The use of such immature oocytes for ICSI is unadvisable due to the asynchrony between sperm entry and oocyte nuclear maturity.

The importance of the presence of a detectable MS in the oocyte cytoplasm prior to ICSI has been extensively described in several studies [68–75]. MS visualization has been associated with oocyte fertilization rate, embryo development, and blastocyst progression. However, the percentage of oocytes functionally superior, with a positive MS at visualization, varies substantially between the different studies, between 60 and 90% [69, 70, 72]. These differences have been related to different factors that get involved during laboratory and clinical practices [76].

When studying the MS with the Spindle View systems, these factors must be taken carefully into account in order to keep under control those that may interfere with the visualization of the spindle. First is temperature control during oocytes handling [68, 77]. Secondly, the absence of MS may be due to unfavorable culture conditions and environmental changes [78, 79]. Third factor is the MS orientation during Pol-Scope visualization [70, 72, 73]: 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 axis [64, 70, 71].

It has been recently shown, with time-lapse cinematography (Octax EyeWare MX), that at the end of the first meiotic division, the MS takes approximately 40–60 min to reform [67]. Therefore, at least in some cases, the absence of MS signal is due to the oocyte maturation process. Most of the oocytes display the meiotic spindle starting at 38 h after hCG administration, and thereafter [72].

Polarized light microscopy allows also the evaluation of the appearance (length, shape) and the retardance of the MS in living oocyte. These parameters (Table 12.2) may be considered during oocyte grading and for oocyte selection together with the other morphological parameters that are usually observed with the conventional light microscopy. Although promising, the validity of this approach has not been proven yet, since discrepancies between MS retardance and normal spindle organization and chromosome configuration assessed by confocal microscopy have been reported [80]. This technique is also expensive (due to the cost of the

instruments and the time necessary for the additional treatment) and potentially dangerous for the oocytes that may be exposed to injurious environmental conditions during observation. Only specialized laboratories equipped with chamber that allow temperature and atmosphere control on the inverted microscope could, in our opinion, benefit from this technology in the routine work.

Cytoplasmic Aspect

Nuclear maturity is only part of the oocyte maturation process. To acquire full competence for subsequent fertilization and development, the oocyte has to undergo to nuclear and cytoplasmic maturation in a coordinate manner. Disturbances or asynchrony of these two maturation processes are believed to result in a variety of oocyte morphological abnormalities [81–83]. Accordingly, cytoplasmic (vacuoles, refractile bodies, increased cytoplasmic granularity, smooth endoplasmic reticulum clusters) and extracytoplasmic (abnormal zona pellucida, large perivitelline space, abnormal, fragmented, or degenerated IPB) defects potentially reflect oocyte developmental impairments [36, 48, 50, 84–86].

Some authors have suggested that despite normal fertilization and early embryo development that can be achieved in oocytes with "abnormal" cytoplasmic morphology, the resulting embryos have a lower implantation potential [43, 87].

Granular cytoplasm and, in particular, centrally located granular area have been associated with poorer zygote quality and poorer embryo quality [39, 50]. These cytoplasmic alterations may thus be, as suggested, a sign of oocyte immaturity [39]. In agreement with other studies [44, 48, 88], when we investigated the relationship between MII oocyte morphology and ICSI outcome [50], we observed that also oocyte vacuolization has a detrimental effect on fertilization rate. Furthermore, no fertilization has been described when vacuole size above 14 μm was present in the injected oocyte [48, 88]. It is believed that vacuoles arise either spontaneously [89] or by fusion of preexisting vesicles derived from smooth endoplasmic reticulum and/or Golgi apparatus [90]. How vacuolization affects pronuclear formation is still unclear. Probably, the presence of these cytoplasmic inclusions interferes with the cytoskeleton function [48]. A negative effect of these structures on the metaphase II meiotic spindle has also been suggested [89].

Meriano et al. [91] and Otsuki et al. [92] have shown that the presence of smooth endoplasmic reticulum clusters in the oocytes significantly impairs embryo development and implantation. In regard to the presence of SER in the oocyte cytoplasm, we were unable to identify a relationship between the presence of this abnormality and fertilization rate and pronuclear/embryo quality. However, because of the very low number of oocytes treated (only 0.5% of oocytes displaying this feature), no real conclusion can be drawn regarding this particular aspect.

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).

First polar body morphology has been suggested as a possible indicator of fertilization and embryo quality after ICSI [47, 66, 93–95]. Embryo transfers selected on the basis of I polar body morphology have been associated with increased implantation and pregnancy rates [93]. However, recent studies have failed to demonstrate any relationship between I polar body fragmentation and embryo implantation rate [66, 94]. Moreover, it has been shown that the aneuploidy rate of the metaphase II oocytes is unrelated to the morphology of the first polar body [96].

In our hands, the presence of an abnormal first polar body (degenerated and/or large) in the metaphase II oocytes analyzed was relatively rare (4.4%). However, this feature was associated with a significantly reduced fertilization rate. It has been suggested that degenerated polar body may reflect an asynchrony between nuclear and cytoplasmic maturation [81], which would explain the lower ability of the cell to support pronuclear formation after ICSI. Furthermore, a correspondence between the presence of large polar body and poor embryo development was already described [45, 66, 93]. According to animal experiments [97], 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 [66]. The polar body morphology, and in particular degenerated and large polar bodies (but not fragmented), may be thus considered a marker of oocyte maturation disturbances [66, 97]. Nevertheless, in our hands, once fertilized, these oocytes did not show an impaired developmental potential in vitro.

A large perivitelline space in the injected oocytes significantly affected fertilization rate and pronuclear morphology [50]. This observation may be ascribed to an overmaturity of these oocytes at the time of ICSI [49].

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. These oocytes contain an extra set of chromosomes and therefore two meiotic spindles when observed at the polarized light microscopy (Fig. 12.4). It has been also described that all embryos generated from giant oocytes are chromosomally abnormal, but they may have a normal cleavage and development to blastocyst stage [98]. The transfer of these embryos could thus increase the risk of undesired miscarriages [98].

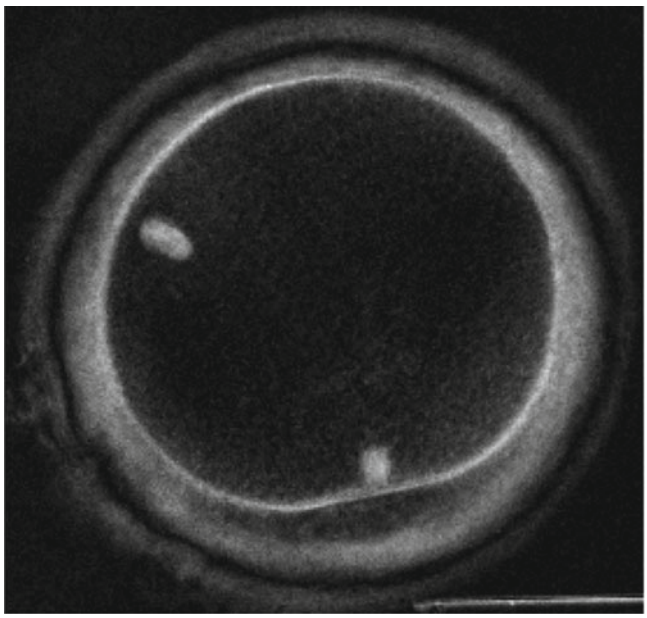

Fig. 12.4 Giant oocyte with two detectable meiotic spindles

Possible Damage to the Oocyte

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 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 follicles aspiration and the high concentration of hyaluronidase used for cumulus cells stripping as well as oocyte pipetting, may activate mature oocytes and induce parthenogenetic activation [99, 100].

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 [100]. Van Steirteghem et al. [101] were able to reduce the activation rate to 3% by employing lower concentration (78 IU/mL) of enzyme. Moreover, Joris et al. [102] 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 millilitre was used. This finding would justify the use of lower concentration of enzyme.

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 the cumulus cells may impair the microtubule frame 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 [70].

The use of polarized light microscopy may be helpful in the assessment of the position of the MS in regard to the IPB. It should be underlined that the MS is not only involved in chromosome segregation but it is also a key organelle in the creation of the IPB. MS position at the very periphery of the cell, attached to the oolemma cortex [103], 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 [69, 70]. 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 [70]. 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 [71]. It is thus advisable to perform oocyte denudation in gentle manner.

Besides affecting ICSI outcome, a possible drawback of IPB displacement is the potential injury that the MS may undergo 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 passageway during sperm injection and hence to mechanical damage. In view of this fact, the Pol-Scope may be a helpful tool during ICSI, allowing the correct orientation of the MS (and not of the IPB) as far as possible from the injection pipette entry spot.

Moreover, it has been widely shown that oocyte exposure to even slight temperature variations dramatically injures the MS structure by inducing microtubules disassembly [68, 104–107]. 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.

Mechanical Stress

In order to avoid damage to the oocytes, a meticulous care should be taken during the mechanical removal of cumulus cells. In fact, an 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 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.

Enzyme Type and Concentration

Hyaluronidase or Coronase

Traditionally, the denuding procedure is performed by using 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 [108]. The use of a plant enzyme preparation containing citric acid and papaine as well as NaCl and phosphate (Coronase) has been proposed as an alternative to animal extracted hyaluronidase. Because of the different action mechanism 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 [109]. 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 [109].

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 1,000 μm diameter has also been shown to be able to denude mature oocytes efficiently [24].

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 circumvent problems and concerns associated with the animal origin. When De Vos et al. [110] conducted a randomized sibling-oocyte study in order to validate the effectiveness of recombinant human hyaluronidase vs. bovine-derived hyaluronidase, they observed comparable results in terms of oocyte survival and fertilization. Evison [111], who observed a significantly increased rate of normal fertilization coupled to a significantly decreased rate of oocyte damage when the recombinant form of the enzyme was used, confirmed these findings. Following up these observations, the human recombinant form of the enzyme may represent a safer, quality-controlled but likewise effective alternative.

Influence of Microenvironment Condition

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 the pH and the temperature, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered media and temperature-controlled humidified chambers are employed to maintain these parameters in a physiological range.

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 [105]. 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 [105].

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°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.

CO_2 Concentration

Dale et al. demonstrated that the baseline intracellular pH (pHi) of human egg is 7.4 ± 0.1 [112]. Unlike germinal vesicle that is endowed with a robust mechanism of pH regulation,

denuded metaphase II oocytes lacking of HCO_3^-/Cl^- and Na^+/H^+ functioning exchangers do not exhibit the ability to control and regulate intracellular pH [113]. It is only 2–3 h after fertilization that the oocyte initiates to recover the exchanger activity and the consequent ability to regulate its pH. To preserve oocyte homeostasis and avoid that pHi fluctuations that may impair developmental competence, HEPES-buffered media are usually employed during the oocytes handling outside the incubator. However, media containing bicarbonate/CO_2 buffers would be preferable, since, although requiring controlled chambers to maintain a 5–7% CO_2 atmosphere, they circumvent 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 that is essential to the functionality of HCO_3^-/Cl^- exchanger and thus the HCO_3^--dependent mechanism that oocytes utilize to defend against acidosis.

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 and fertilization, as well as the implantation process, are modulated by a ROS formation [114–116], 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 female reproductive tract, embryos encountered a decreasing oxygen gradient. Moreover, oxygen levels in oviduct and uterus of several species are considerably lower than in the atmosphere (1.5–6 vs. 21%).

Therefore, it has been suggested that the employment of low oxygen tension (5%) within the incubation atmosphere may improve mammalian embryo development [117–120]. Even if the precise O_2 level is still unknown and may be developmental stage dependent, the low oxygen level should be a principal for all mammalian embryo culture systems including humans.

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 [121] or reducing oxidative stress [122]. A study involving sibling oocytes investigated the actual effect of homologous adhering cumulus cells, resulting in an improvement in terms of preimplantation development when oocytes were cultured with attached cumulus cells [123].

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 [124, 125].

Future Direction and Improvement

The involvement of automated systems in IVF procedures is attracting increasing attention. A microfluidic device has been proposed to remove cumulus cells automatically, aiming to minimize physical stresses related to this procedure [126]. The system entails pressure-driven flow to obtain a correct positioning and transit of CCOC into microchannel that allow 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.

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Assessment of Oocyte Quality

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Abstract

After oocyte collection for assisted reproductive technologies, the oocytes display various aspects of maturation, integrity and viability. As there is still a lack of reliable and rapid biochemical, or molecular marker of their status, the best modality for evaluation of oocyte quality still remains to be direct microscopic observation of morphology. Morphological assessment of human oocytes retrieved for ART remains to be a major tool in predicting in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) outcome and should be further developed. There are no clear and well-defined criteria for oocyte morphology evaluation as there are no widely accepted grading systems in effect. Despite the fact that clear decisions for all types of morphological differences of human oocytes cannot be based upon data, some recommendations for specific abnormality types can be proposed. It can be concluded that morphological evaluation of the oocyte should be coupled with a detailed evaluation of the resulting embryo to reach a valuable conclusion regarding implantation and pregnancy. Certain morphological characteristics indicate a high likelihood of abnormality, and caution should be exercised when transferring embryos derived from such oocytes.

Keywords

Morphological assessment of human oocytes • IVF • Ovarian stimulation and oocyte quality • Gigantic oocytes and nuclear and cytoplasmic maturation • Oocyte quality evaluation

The identification of the viable human metaphase II oocyte is a controversial issue that has been subject to much debate in the last decade. The intricate and complex events during follicular maturation determine the capacity of the oocyte to undergo normal fertilization and subsequent embryonic development. Complete physiological maturation requires nuclear and cytoplasmic changes that need to be coordinately completed to ensure optimal cellular conditions. The asynchrony of nuclear and cytoplasmic maturation events may compromise oocyte quality, resulting in different oocyte dysmorphisms.

Starting from retrieval, cumulus–corona complex morphology and its relation with nuclear and cytoplasmic maturity of the oocyte have been studied over the years and correlated with different grading systems identified in the literature. With the application of intracytoplasmic sperm injection (ICSI) procedures that require mechanical and enzymatic elimination of the cumulus–corona cell layer, it became possible to identify and classify in detail the morphological deviations of the metaphase II oocyte. These deviations are either extracytoplasmic or cytoplasmic or a combination of both. Extracytoplasmic abnormalities include irregular-shaped oocytes, zona pellucida abnormalities, first polar body dysmorphisms and perivitelline space (PVS) deviations. Reported cytoplasmic abnormalities are cytoplasmic granularity and homogeneity, cytoplasmic viscosity

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and the presence of cytoplasmic inclusions (dark incorporations, spots, refractile bodies, vacuoles and smooth endoplasmic reticulum clusters [sERCs]).

While we are all eagerly awaiting for the most accurate and non-invasive way of assessing human oocyte viability, there is still no routinely applicable technique or analytical device yet available to fulfil this goal. Hence, in vitro fertilization (IVF) clinics worldwide continue to select oocytes based on their light-microscopic morphology, thus making oocyte morphology evaluation schemes variable and highly subjective.

Morphological variations of the oocyte may result from intrinsic factors, such as age [1] and genetic defects, or extrinsic factors, such as medications used for controlled ovarian stimulation (COS) and the ovarian response to COS [2, 3].

This chapter aims to clarify the effect of different parameters on the morphological changes of the oocyte. These changes include; intrinsic factors (age [1], genetic defects) and extrinsic factors (COS protocols and ovarian reserve [2, 3]). There uses identifies morphological variations in oocyte evaluation schemes, as well as discuss morphological markers of oocyte quality/viability in relation to oocyte morphology. They are also used in attempting to determine whether morphological evaluation of the oocyte can be utilized for predicting the implantation potential of the derived embryo.

Effect of Ovarian Stimulation on Oocyte Quality and Morphological Appearance

Oocyte quality has been shown to be affected by ovarian stimulation regimens and hormonal environment changes which may result in the maturation of abnormal oocytes [4]. Majority (60–70%) of the oocytes retrieved from stimulated cycles exhibit one or more abnormal morphological characteristics [5].

There are very scarce data regarding the effect of different stimulation protocols, different gonadotropin preparations, the dosage and duration of medications employed for ovarian stimulation and oestradiol concentrations on the maturity and competence of oocytes. It has been recently shown that, no matter which stimulation protocol is used, pharmaceutical preparations of human gonadotropins used to induce the multiple follicular growth might influence the growing oocyte [6, 7]. Excessive ovarian stimulation and the resulting hyperestrogenic milieu may have detrimental effects on oocyte quality [8, 9]. This investigation suggests that amongst the cohort of recruited follicles, only the most sensitive to stimulation is likely to yield better quality embryos, whereas all the additional oocytes resulting from maximal stimulation might be of impaired quality. Alternatively, the reduction in pregnancy rate observed after maximal stimulation might be due to a direct effect of high serum oestradiol concentrations on the oocytes [10, 11] or on endometrial receptivity [11, 12]. Earlier studies suggested that high concentrations of LH during follicular phase of stimulation could have a negative impact on oocyte quality, pregnancy rate and the incidence of miscarriage [13–15]. Several studies examined the effect of polycystic ovarian syndrome on the maturation and quality of the oocyte, but the results were controversial [16]. Data from women with PCOS suggest that oocyte and embryo quality and implantation may be impaired in these patients treated with assisted reproduction [17]. This may be due to impaired gamete quality resulting from the high oestrogenic milieu or intrinsic problems. However, not all results are in agreement, and clinical outcome of PCOS patients appears to be equivalent to other infertility factors [5].

Most of the studies published so far examining the effect of clinical parameters on oocyte quality have been dependant upon oocyte maturity and quality rather than morphology [5]. Imthurn et al. [18] examined the effect of highly purified follicle stimulating hormone (HP-FSH) on nuclear maturity and morphological appearance of the oocyte in couples undergoing ICSI and compared the results with human menopausal gonadotropin (HMG) stimulation. Authors did not find any difference amongst the groups in relation to patient age, duration of stimulation, number of aspirated oocytes or maturity of the oocyte–cumulus complex. Rashidi et al. [2] also showed that nuclear maturity, oocytes with abnormal zona, polar body or cytoplasmic morphology were similar between the group of patients stimulated with either HMG or recombinant FSH.

Otsuki et al. [19] showed that the risk of producing oocytes with an aggregation of the sER is increased in patients with high levels of serum oestradiol on the day of human chorionic gonadotropin (HCG) administration. As the application of a short protocol seems to favour formation of sER clusters, this finding indicates that ovarian stimulation may directly influence oocyte appearance and quality, e.g. through recruitment of follicles that in vivo might have become atretic.

Similarly, PVS granularity, a dysmorphism that, not interacting with fertilization, cleavage behaviour and pregnancy outcome was increased in patients exposed to higher doses of gonadotropins [20].

Effect of Oocyte Morphology on Embryo Development and Implantation

Oocyte maturation consists of two separate processes: nuclear and cytoplasmic maturation, both of which should take place in a well-coordinated and synchronized manner in order to guarantee adequate oocyte quality [20]. In this context, nuclear maturity refers to the resumption of meiosis

and the progression to metaphase II, the natural arresting point prior to ovulation [21]. Furthermore, full physiological maturation appears to require nuclear and cytoplasmic changes that need to be coordinately completed to ensure optimal cellular conditions [22]. The presence of a normal nuclear genetic material will not guarantee the potential of the oocyte to develop into a healthy embryo and foetus. While resumption of the first meiotic division takes place and the nucleus enters metaphase II stage, synchronously cytoplasmic maturation goes on achieving zonal ability to release calcium and cortical granules, mitochondrial changes, protein synthesis and cytoskeletal changes [23]. Absence of nuclear cytoplasmic coordination is seen in germinal vesicle (GV)-stage meiotically competent oocytes recovered from follicles at different stages of development, where the ability to support preimplantation development is achieved progressively during antrum formation [24]. The oocyte nuclear compartment is directly involved in the LH-mediated changes. These changes resume the meiotic process, which is undergoing germinal vesicle breakdown. When it reaches metaphase I and becomes characterized by the absence of the first polar body extrusion, some of the intermediate eggs may be fertilizable and arresting at the metaphase II (MII) stage. Disturbance or asynchrony of these two processes has been shown to result in different oocyte morphological abnormalities. A mature nucleus and cytoplasm are regarded as equally important for embryo development, and each is closely associated with oocyte morphology [25–27].

Correlation of Oocyte–Cumulus–Corona Complex Morphology with the Quality and the Maturity of the Oocyte

Apart from inducing the rupture of the follicle wall and triggering the conversion of the follicle into the corpus luteum, LH stimulation causes precise changes in the oocyte and the surrounding cumulus cells. Cumulus cells produce a viscoelastic extracellular matrix that accumulates in the intercellular spaces. This matrix, whose most characteristic component is hyaluronic acid, is organized into a mesh-like network that allows the preservation of a close contiguity between cumulus cells and the oocytes, despite the partial or total loss of intercellular coupling and contact between these cells [24].

Immature oocyte–cumulus–corona complex (OCCC) displays an unexpanded cumulus and a dense corona forming a compact layer of cells adhering to the zona pellucida of a prophase I (germinal vesicle-bearing) oocyte. The ooplasm cannot be seen through the cumulus mass associated with small parietal granulosa cells which appear in compact clumps [28]. In stimulated cycles, it is common to recover OCCC that display some degree of cumulus expansion but a compact corona layer or even an expanded cumulus–corona complex and nevertheless contain immature prophase I oocytes. If they are not recognized at harvest and are inseminated immediately, they will result in an absent or delayed fertilization. Under the stereomicroscope, a typical mature preovulatory OCCC displays an expanded radiating corona surrounded by the loose mass of cumulus cells, macroscopically visible [28] and mucified layer, due to the active secretion of hyaluronic acid in support of positive effects of cumulus–corona cells on oocyte development [29]. The sparse structure of these cells allows identification of the oocyte with a spherical, homogeneous ooplasm and sometimes the first polar body extruded in the PVS. Usually, however, cumulus and/or corona layers are dense in appearance and at times darkened, and the polar body cannot be observed [28].

Table 13.1 Grading system for cumulus–oocyte complexes

Grade 1 (A)	Absent to sparse cumulus cells and 1–3 layers of corona cells
Grade 2 (B)	Dense cumulus cells and tightly packed corona cells
Grade 3 (C)	Expanded, fluffy cumulus cells and expanded corona cells
Grade 4 (D)	Expanded, scanty cumulus cells and expanded, often partially lost corona cells

Post-mature OCCC are believed to arise from cycles where there has been a premature attenuated LH surge or a delayed HCG administration. The cumulus displays clusters of darkened cells, while the corona is usually dark and tight. Degenerative or atretic OCCC, which form 3% at recovery, exhibit clear signs of abnormalities, e.g. oocytes with a dark and/or contracted irregular ooplasm, disrupted zona pellucida or empty zona surrounded by a retracted cumulus mass. It is recommended to discard these groups of OCCC and yielded oocytes from further culture and insemination [28].

There are very few studies in the literature that correlated OCCC morphology and oocyte quality since oocyte maturity at the time of retrieval is difficult to assess being obscured by a large cumulus mass infiltrated with abundant hyaluronic acid. Ng et al. [30], who used a modified grading system adapted by Wolf [31] (Table 13.1), showed that mature grade 3 cumulus–oocyte complexes (OCCC) were associated with higher fertilization rates. The pregnancy rate was higher in cycles when >50% of retrieved OCCC were grade 3 compared with cycles where ≤50% of OCCC were similarly graded [5].

A more recent study graded oocyte maturity on a scale from 1 to 5 based on the morphology of the ooplasm, cumulus mass, corona radiata and membrana granulosa cells [32] (Table 13.2). Grade 1 (mature) oocytes were included in the first group, whereas the second group consisted of oocytes graded from 2 to 5 (immature). This study showed that mature oocytes yielded higher fertilization rates. Although

Table 13.2 Grading system for oocyte maturity

Grade 1 (mature or preovulatory)	Expanded cumulus, very radiant corona, distinct zona pellucida, clear ooplasm, expanded well aggregated membrana granulosa cells
Grade 2 (approximately mature)	Expanded cumulus mass, slightly compact corona radiata, expanded well aggregated membrana granulosa cells
Grade 3 (immature)	Dense compact cumulus if present, very adherent compact layer of corona cells, ooplasm if visible with the presence of the germinal vesicle, compact and non-aggregated membrana granulosa cells
Grade 4 (post-mature)	Much expanded cumulus with clumps, radiant corona radiata yet often clumped, irregular or incomplete, very visible zona, slightly granular or dark ooplasm, small and relatively non-aggregated membrana granulosa cells
Grade 5 (atretic)	Rarely associated with cumulus mass, clumped and very irregular corona radiata if present, very visible zona, dark and frequently misshapen ooplasm, membrana granulosa cells with very small clumps of cells

cleavage rates were similar in both groups, the percentage of poor morphology day 3 embryos from the immature group was significantly higher than from the mature group.

However, most of the studies published in the literature are based on the utilization of OCCC morphology for IVF cases where OCCC were not removed before fertilization check. The utilization of OCCC morphology for determination of good quality and mature oocytes is less likely to be applied nowadays as more and more IVF laboratories prefer microinjection as the method of fertilization, during which the maturity of the oocyte can more precisely be assessed following enzymatic and mechanical digestion of the OCCC.

A recent study by Engman et al. [33] similarly showed that for women undergoing ICSI, assessment of OCCC (oocytes–cumulus–corona complex) morphology is pointless; since there is no correlation between OCCC morphology, fertilization and cleavage rates. For conventional IVF, OCCC morphology may be useful for gamete selection if the couple wants to electively limit the number of oocytes to avoid creating surplus embryos for cryopreservation.

Morphological Dysmorphisms of the Metaphase II (MII) Oocytes and General View of Cytoplasmic and Extracytoplasmic Abnormalities

Removal of cumulus cells from oocytes of patients undergoing ICSI allows a more detailed observation of the 60–70% of all oocytes' morphological characteristics [24, 34]. Mature and good quality metaphase II oocyte is defined as: clear, moderately granular, homogenous and translucent cytoplasm without inclusions, small PVS, a clear to colourless and regular zona pellucida, perfectly spherical shape and an intact PBI [21, 24, 35–37]. However, the majority of the oocytes retrieved after ovarian hyperstimulation exhibit one or more variations of the described "ideal" morphological criteria [21, 35, 36, 38, 39]. Even in the very early years, it had been shown that 13% of unfertilized oocytes after IVF harboured morphological abnormalities [40].

Morphological deviations of metaphase II oocytes can be classified as intracytoplasmic and extracytoplasmic abnormalities. Most frequently observed cytoplasmically morphological variations are changes in colour, granularity of the cytoplasm presence of cytoplasmic inclusions such as vacuoles and sERCs and cytoplasmic incorporations. Extracytoplasmic variations are deviations from normal visualization of PVS, zona pellucida colour, first polar body morphology (fragmented or degenerated) [34, 41] and shape abnormalities [25, 35, 41, 42].

Cytoplasmic Abnormalities

Although the effect of cytoplasmic abnormalities on the clinical outcome had been controversial, evidence-based data on severe abnormalities agree on the point that embryos derived from oocytes with severe cytoplasmic abnormalities have reduced viability and implantation potential. One of the earliest studies by Xia et al. had shown that cytoplasmic abnormalities, such as dark cytoplasm, refractile bodies, dark incorporations, spots and single or multiple vacuolization and granulation in the cytoplasm, were correlated with fertilization and embryo quality [24, 35, 36]. The study by Serhal et al. also showed significantly lower fertilization rates, embryo cleavage rates and lower embryo quality in the group of oocytes with cytoplasmic inclusions [36, 43]. Therefore, it has been indicated even in the early years that cytoplasmic texture appears to be a very important characteristic for oocyte evaluation, and these cytoplasmic alterations may be a sign of oocyte immaturity [44].

Despite the presence of normal genetic material, ooplasmic factors play an important role in the fertilization process that could be compromised by cytoplasmic abnormalities [45]. Deficiency in cytoplasmic maturation could compromise all processes that prepare the oocyte for activation, adequate fertilization and embryo development. In addition, a disturbance or asynchrony of these two processes has been shown to compromise oocyte quality, resulting in different oocyte dysmorphisms [21, 44].

Given the data in the literature, it may be concluded that only severe cytoplasmic defects such as organelle clustering/centrally located granulation, appearance of sERCs and certain types of fluid-filled vacuoles should be considered as abnormalities, whereas slight deviations from the normal cytoplasmic structure should be accepted as normal oocytes with a phenotypically heterogeneous cytoplasm [46].

Cytoplasmic Granularity, Centrally Located Granulation and Cytoplasmic Viscosity

Cytoplasm changes, which accompany the oocyte growth, include mRNA transcription and protein synthesis [47, 48]. These processes are necessary for the meiotic maturation of oocyte, activation of the zygotic genome and blastocyst formation [49, 50]. Oocyte granularity is correlated with the localization of mitochondria and may represent domains of high ATP request that are necessary for the normal development of embryos [51, 52]. Slight or moderate granularity has been accepted as a normal feature of oocytes. It is conceivable that an increased viscosity of the cytoplasm may constrain cell organelles and/or pronuclei in their movement preventing the zygote from achieving alignment of both pronuclei or alignment of pronuclei with respect to the polar bodies, thereby severely impairing polarity and further preimplantation development [53, 54]. However, dense granularity focused in the centre of the oocyte is considered as a dysmorphism [5]. In correlation with these, Balaban et al. [42] showed similar fertilization rates and embryo quality in the group of embryos derived from oocytes with slight granular cytoplasmic appearance. Kahraman et al. [44] who studied condensed centrally located granulation in the oocyte cytoplasm also showed similar fertilization rates and embryo quality (4.2%) from embryos derived from such oocytes. However, the ongoing pregnancy rate was significantly decreased with higher abortion rates. It was also demonstrated in the same study that centrally located granulation is correlated with high chromosomal aneuploidy possibly being the reason for high abortion rates.

A more recent study by Rienzi et al. [55] showed that although fertilization rates of oocytes with cytoplasmic granularity or centrally located granular area are not affected, pronuclear morphology and embryo quality were affected by the presence of a centrally located granular area [55]. The authors suggested an MII oocyte morphological score (MOMS) including mainly cytoplasmic abnormalities and demonstrated that this grading system is predictive of the clinical outcome.

Cytoplasmic granulation and cytoplasmic viscosity appear to be in close correlation. It has been reported that granular areas are more viscous than the surrounding cytoplasm [56]. There is a lack of markers of increased cytoplasmic viscosity [57]. Ooplasm of higher viscosity is more likely to adhere to the spike of the injection pipette which could be seen in a limited number (~0.5%) of injections after withdrawal of the glass tool. Persistence of the injection funnel after ICSI reflects a deficiency in cytoplasmic texture. Flux characteristics of cytoplasm are altered in the more viscous type which does not allow the oocyte to restore its original spherical shape as fast as seen in gametes with a more aqueous ooplasm. Since all oocytes involved show an injection funnel during ICSI, the observed difference in degeneration rate may be explained by a difference in cytoplasm fluidity. In other words, increased viscosity of the cytoplasm (persistent funnel) may keep it from leakage, whereas oocytes with more aqueous cytoplasm tend to leak more frequently after ICSI [56].

Another study by Loutradis et al. [58] showed that cytoplasmic viscosity impairs embryo quality, blastocyst formation rate, blastocyst quality, as well as the pregnancy and implantation rates [58].

Vacuolization

The presence of vacuoles or inclusions within the cytoplasm is considered to be a severe abnormality [19, 40, 59]. How cytoplasmic inclusions such as deficiency in cytoplasm fluidity and ooplasm vacuolization affects fertilization and further embryonic development processes remains unclear [60, 61]. This phenomenon could be related to cytoskeletal function and MII meiotic spindle structure [41, 60, 62]. Cytoplasmic defects lead to detrimental effects to pronuclear formation, as the cytoskeleton cannot function properly and the MII spindle can be displaced from its polar position [4].

Biological basis and possibly a genetic cause may result in either uncontrollable endocytosis or poor exocytosis and consequent vacuolar formation. Spindle visualization showed that macro vacuoles were not associated with spindle displacement. The presence of vacuoles and vesicles in the oocyte cytoplasm influenced the fertilization characteristics of oocytes negatively, even in the presence of top scores for other morphological characteristics [63]. De Sutter et al. [36] found a severely reduced fertilization rate in vacuolated oocytes (40%). Esfandiari et al. [46] also reported significantly lower fertilization rates for the group of oocytes with multiple vacuoles when compared with the oocytes with a single vacuole in the cytoplasm. Ebner et al. [64] recently analysed the actual influence of vacuolization (time of first appearance, number and size of vacuoles) on preimplantation embryo development up to the blastocyst stage. The authors [64] found that vacuoles in 3.9% of oocytes at collection, of which 66% had single, 21.3% had double and 12.7% had multiple vacuoles. Other studies [35, 37] suggest a slightly higher oocyte vacuolization rate (5, 7 and 12.4%, respectively).

Vacuoles arise either spontaneously [41] or by fusion of preexisting vesicles derived from SER and/or Golgi apparatus [65]. There was a significant relationship between the size

of vacuoles (cut-off value 14 μm) and fertilization. The mean diameter of vacuoles in fertilized oocytes was 9.8 μm, compared with 17.6 μm for non-fertilized oocytes. The presence of membrane-bound vacuoles in the cytoplasm appears to be associated with a reduced fertilization rate [36, 60, 64].

It was demonstrated in the same study that vacuoles have a detrimental effect on embryo development as well, with significantly decreased rates of blastocyst formation from the group of embryos that were derived from oocytes with at least a single vacuole in the cytoplasm. In correlation with the previous findings, Rienzi et al. [55] also showed significantly lower fertilization rates in vacuoled oocytes. Besides lower fertilization rates, pronuclear morphology and embryo quality were reported to be similar between the group of fertilized oocytes with vacuolated cytoplasm or normal cytoplasmic texture. However, this study did not segregate the group of vacuolated oocytes according to their number or sizes.

It is very likely that a larger vacuole or multiple vacuoles will cause a much more detrimental effect to the oocyte than a small vacuole, since a larger portion of the cytoskeleton (e.g. microtubuli) cannot function as it is supposed to [21]. It has previously been demonstrated that extremely large cytosolic vacuoles physically displace the MII spindle from its usual polar position and thus might cause fertilization failure, as well as zygotic and embryonic arrest [41].

Macro vacuoles present within the ooplasm appear to have distorted oocyte cytoskeletal structure to such an extent that physiological processes involved in fertilization and embryogenesis were impaired, including sperm–oocyte signalling, sperm binding, meiotic resumption and embryonic cleavage [19, 41].

A novel solution to this problem could be to attempt the removal of the massive cytosolic vacuoles using modern micromanipulation techniques. However, vacuole drainage could result in a reduction in cytoplasmic volume, which in itself could be detrimental to embryonic development. Puncture and drainage of the vacuoles could also result in the leakage of intravacuolar fluid, which could contain toxic waste products that should have been exocytosed, into the surrounding ooplasm [66].

Appearance of Smooth Endoplasmic Reticulum Clusters

sERCs can also be defined as pronucleus sized slightly bulged/flat translucent vacuoles localized in the centre of the cytoplasm at the metaphase II stage and can be easily distinguished under inverted microscope from regular type of fluid-filled vacuoles (identical with perivitelline fluid [41]).

Intrinsic oocyte-specific defects of important molecular and cellular activities exist that are hardly detectable using conventional microscopic techniques. Electron microscopic studies stressed that cytoplasmic organization of normal-appearing oocytes is characterized by an apparently random distribution of cell organelles. Oocytes showing deviations in fertilization or further development often display atypical distributions of cellular components [41]. A disk-like assemblage of the sER would represent such a cytoplasmic defect that could adversely influence competence [40]. It is presumed that these clusters arise by dilatation and fusion of sER saccules during maturational process of the gamete [41]. The relatively low frequency of this oocyte dysmorphism has kept several authors from analysing it separately [42, 43]. Others, however, scored between 1% and 3% of all analysed gametes to be sER cluster positive [37, 41, 60, 67].

These figures may be somewhat underestimated, since it became apparent from the findings of Otsuki et al. [19] that smaller clusters (e.g. 2–5 ptTi) cannot be detected without the help of an electron microscope. These smaller aggregations were exclusively found in presumed sER-negative oocytes (on the basis of light-microscopic analysis) of sER cluster-positive women [19], and thus, no bias was introduced into statistical analysis, as probably no sER-positive patients were missed. In addition, these smaller aggregations were exclusively situated in the cortical area of the ooplasm, and it is not clear whether they have the same detrimental effect as larger accumulations near the centre of the oocyte. lt should be noted that the smallest aggregation detected in this prospective study was 5.1 μm in size and found in an egg with these clusters. Otsuki et al. [19] also showed that sER clusters are an exclusive anomaly of MII oocytes (not detected in prophase or metaphase I gametes) and tend to grow in diameter before disappearing approximately 16–20 h post-ICSI.

Otsuki et al. [19] studied the relationship between pregnancy outcome and sERCs in MII human oocytes. The presence of sERC in the cytoplasm significantly impaired embryo quality and was associated with a lower chance of conception even in sERC (oocyte-derived embryos from the same cohort that were transferred along with embryos derived from sERC (+) oocytes). Because Ca^{2+} release from sER plays a pivotal role in oocyte maturation, fertilization and early embryonic development [68] studies of Ca^{2+} signalling in sERC (+) oocytes may contribute to understanding of the cause and the effect of sERC [5]. In contrast, no relationship was found between the presence of sER in the oocyte cytoplasm and fertilization rate and pronuclear/embryo quality [55]. Besides these effects on embryo development and clinical outcome, they reported that one baby born in sERC-positive cycles was diagnosed with Beckwith–Wiedemann syndrome, a model imprinting disorder resulting from mutations or epigenetic events affecting the imprinted genes on chromosome 11p15.5. Questions arise as to whether inheritance of this disease is causally related to the use of assisted reproduction techniques [69, 70]. At present, there are no data concerning the relationship between sERC and the

genomic imprinting defect. Currently, the correlation between sERC and imprinting disorders is not clearly known. It would be important to examine the unusual distribution pattern of the sERC formation that may be involved in the abnormal regulation of Ca^{2+} signalling.

Ebner et al. [71] recently showed that the appearance of sERC is significantly related to duration and dosage of the stimulation. Fertilization (58.9%) and blastulation rates (44.0%) were significantly lower in affected ova compared with unaffected counterparts (77.4 and 87.8%, respectively). Pregnancies in women with affected gametes were accompanied by a higher incidence of obstetric problems ($p<0.01$) leading to a non-significant trend towards earlier delivery and significantly reduced birthweight ($p<0.05$). This study recommended avoiding transfer of embryos/blastocysts derived from SER cluster-positive gametes and suggested to inform patients that even transfer of sibling oocytes without this anomaly involves a higher risk of detrimental outcome.

Meriano et al. [59] showed that the presence of sER in the oocytes significantly impairs clinical pregnancy and implantation rates despite similar fertilization and cleavage rates and embryo quality. Very importantly intracytoplasmic organelle clustering was the only significant repetitive abnormality in between other cytoplasmic and extracytoplasmic abnormalities that was found to be a negative predictor of pregnancy and implantation after ICSI.

It is clear that sERCs in human oocytes require future studies due to important functions of the sER in oocyte maturation and Ca^{2+} signalling for embryonic development. More research is needed to define the molecular and cellular mechanisms of organelle clustering [59].

Extracytoplasmic Abnormalities

The effect of extracytoplasmic abnormalities on clinical outcome is unclear. Although most of the published data in the literature did not show detrimental effects, there are still studies reporting controversial results. Extracytoplasmic abnormalities may be a physiological maturation-related phenomenon affected by hyperstimulation protocols and less stringent follicular selection [4]. One of the earlier studies had shown that extracytoplasmic abnormalities such as dark zona pellucida, zona pellucida thickness, large PVS or irregular shape of the oocyte are not related to fertilization rate and embryo quality after ICSI and commented that the oocytes showing such morphological deviations should be regarded as normal [35, 37, 72]. Results vary as to whether distinct oocyte dysmorphisms have any relation to fertilization and development rates, probably due to discordance in the scoring of these dysmorphisms [5]. Increased rate of degeneration after ICSI of oocytes with extracytoplasmic abnormalities is often noted [5, 35, 37, 73]. Although Ebner et al. [62] and Plachot et al. [74] have shown that extracytoplasmic abnormalities of the oocyte did not affect fertilization and embryo quality, both studies reported higher lysis rates after ICSI in the group of oocytes with outer layer abnormalities (fragile oolemma, dark zona pellucida, large PVS and shape irregularity). Outer layer abnormalities may be somehow related to sudden oolemma breakage pattern described by Palermo et al. [75].

Based on most of the published data, it is difficult to comment on only the effect of the extracytoplasmic abnormalities, since the grading systems used were mainly based on numerous factors including at least one additional cytoplasmic abnormality. Xia et al. [35] indicated that the main factor affecting fertilization and embryo quality was the characteristic of the cytoplasm which highly significantly decreased fertilization rates and embryo quality for the group of oocytes with cytoplasmic inclusions even without any extracytoplasmic abnormality. In contrast, there was a slight decrease in the fertilization rate and embryo quality in the group of oocytes without cytoplasmic inclusions but with extracytoplasmic abnormalities (fragmented first polar body, large PVS). Therefore, the main determinant for embryo developmental potential was found to be cytoplasmic properties rather than extracytoplasmic deviations. In line with these observations, Balaban et al. [42] showed that extracytoplasmic abnormalities of the oocyte such as dark zona pellucida, large PVS or shape abnormalities were not associated with a decreased fertilization rate or unfavourable embryo quality after ICSI [5]. It has been shown that the repetition of specific oocyte dysmorphisms from cycle to cycle is a negative predictor of pregnancy and implantation rates in ICSI [59]. However, none of the extracytoplasmic abnormalities such as large or granulated PVS or zonal abnormalities were found to be repetitive from one cycle to another [5].

Evaluation of First Polar Body Morphology

Recent studies investigated a possible correlation between PBI morphology and oocyte competence [76]. It has been claimed that PBI morphology assessment can be used to determine the post-ovulatory age of the oocyte [77]. The PBI morphology has also been suggested as a possible indicator of fertilization and embryo quality after ICSI [78, 79]. Ebner et al. [78] described five different morphological appearances of the first polar body. Grade 1 first polar bodies (round or ovoid and intact) differed from grade 2 first polar bodies (also round or ovoid and intact) in that the former has a smooth surface. Grade 3 (more than two fragments) and grade 4 (broken into two) first polar bodies were defined as fragmented, whereas grade 5 first polar bodies were characterized by their huge appearance being extruded to a similarly large PVS. ICSI to oocytes showing grade 1 and 2 first polar bodies resulted in higher fertilization rates and gave rise to higher quality embryos [39].

Embryo transfers selected on the basis of PBI morphology have been associated with increased implantation and pregnancy rates [78, 80]. Oocytes with an intact PBI of normal size undergo fertilization at a rate comparable to that of oocytes with a fragmented PBI (77.1 and 75.9%, respectively) but produce a significantly lower incidence of fragmentation in day 2 embryos (10.9 and 13.2%, respectively) and a higher blastocyst formation rate (54.9 and 42.2%, respectively) [81]. The presence of an abnormal PBI (degenerated or large) in the MII oocytes analysed was relatively rare (4.4%). However, this feature was associated with a significantly reduced fertilization rate. It has been suggested that a degenerated PBI may reflect an asynchrony between nuclear and cytoplasmic maturation [82]. Ebner et al. [81] showed that embryos with an intact first polar body group were associated with an increased rate of blastocyst formation when compared with the fragmented first polar body group [81].

In contrast to the studies indicated above, more recent studies came up to a conclusion that, as PBI formation is a dynamic procedure, fragmentation rates might differ within hours of culture, and therefore, the prognostic value of its evaluation can only be limited [79, 83, 84]. These studies were also able to demonstrate that there was no significant relation for fertilization rate, embryo quality or clinical outcome [79, 83, 84]. It has been reported in the same studies that PBI fragmentation is not related with the implantation potential of the resultant embryo [79, 83]. The PBI morphology, in particular degenerated and large (but not fragmented) polar bodies, may therefore be considered to be a marker of oocyte maturation disturbance [79, 85].

Verlinsky et al. [84] genetically analysed embryos derived from different 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 huge polar bodies was not analysed [84]. The investigators also reported that the first polar body morphology was not associated with the chromosomally normality of the developing embryo. Verlinsky et al. [84] also detected first polar body morphology grading changes in terms of fragmentation in more than one-third of the oocytes studied. It appeared that morphology of the first polar body changes after a few hours of in vitro culture, and it can vary according to the time during which the observation is carried out. Therefore, the authors concluded that first polar body morphology assessment may not serve as a reliable marker of oocyte quality and competences.

Shape Abnormalities

Since most of the data in the literature examined extracytoplasmic abnormalities jointly, there is insufficient evidence-based analysis on the possible effect of shape abnormalities on embryo quality and viability, as well as the clinical outcome. Balaban et al. [42] reported that the incidence of irregular-shaped oocytes is around 3% within a whole cohort of oocytes retrieved. This study showed that out of 223 injected oocytes with shape abnormality, no significant difference was found in terms of fertilization and cleavage rates and embryo quality when compared with the group of oocytes with normal morphology. More recently, the study by Rienzi et al. [55] also demonstrated that the fertilization capacity of oocytes is not affected by the appearance of shape abnormalities.

Yakin et al. [86] demonstrated that similar fertilization, cleavage rates, cleavage stage embryo quality, blastocyst formation and quality could be obtained from the group of zygotes derived from oocytes with shape abnormalities when compared with group of zygotes derived from oocytes with normal morphology. More recently, Rienzi et al. [55] showed similar rates of fertilization, normal pronuclear morphology and cleavage stage embryo quality for the group of embryos obtained from oocytes with shape abnormalities.

Zona Pellucida Abnormalities

Abnormalities of zona pellucida can be described in different forms such as distorted, multi-layered, pigmented, hairy appearance, thinner or thicker than usual [28] or darker appearance. Zona abnormalities can appear in some oocytes as a "ghost" zona in which the top bilayer appears to detach or pull away from the bottom zonal bilayer [59].

The study by Balaban et al. [42] had shown similar fertilization, embryonic development, clinical pregnancy and implantation rates for the group of embryos derived from oocytes with dark zona appearance when compared with embryos obtained from oocytes with normal morphology [42]. More recently, the same group reported similar cryopreservation rates, and similar blastocyst formation, quality and hatching rates for the group of frozen–thawed embryos derived from oocytes with dark zona pellucida when compared with the frozen–thawed embryos derived from oocytes with normal morphology [87].

Palermo et al. [75] support the hypothesis that the hormonal environment during ovarian stimulation may affect the oocyte quality, e.g. oolemma behaviour as well as zona pellucida appearance. Any deviation from a presumed normal injection procedure (sudden and difficult breakage) could indicate a change in the three-dimensional structure of the outer shell, causing problems during the hatching process [88].

Low expression of the zona proteins by the growing human oocyte may indicate reduced developmental potential. In this respect, a German group [89] used polarization light microscopy to non-invasively analyse the texture and thickness of human zona pellucida by quantitatively measuring retardation magnitude and thickness of the three layers of

the zona. In contrast to the middle and outer layer, the innermost one was significantly thicker and showed a nearly 30% higher light retardation in conception cycles as compared with cycles not resulting in pregnancy [21].

The same authors [89] observed that oocytes, with zona splitting, probably caused by mechanical stress during retrieval or denudation, were exclusively associated with non-conception cycles. An additional explanation would be that in these cases, the patterning of proteins may be temporarily interrupted during formation of the extracellular coat [21]. Though these types of ova usually show an ovoid shape, it has to be noted that the zona is responsible for the dysmorphism, and the oocyte, however, reveals a spherical shape. If both zona pellucida and oocyte are involved in distortion, corresponding embryos run the risk of developmental incompetence. If this shape dysmorphism runs throughout preimplantation development, theoretically, it will result in an atypical elongated embryo [21]. The thickness of the zona pellucida which influences sperm penetration varies from 10 to 31 μm and is not related to the cytoplasm diameter.

The oocytes are fertilized best in vitro when the thickness of the zona pellucida was less than 18.6 μm. The thick zona pellucida (22 μm and thicker) could be an indicator for the use of assisted hatching of embryos produced by ICSI from infertile patients [90]. The thickness of the zona pellucida had no influence on the embryo development after ICSI [91].

Cohen et al. [92] and Palmstierna et al. [93] suggested that patients transferred with embryos with thinner zona pellucida had a better chance of successful implantation and pregnancy compared with transferred with embryos with thicker zona pellucida [91]. Gabrielsen et al. [91] reported in their study that embryos with a minor variation in zona pellucida thickness had an implantation rate about 10%, which increased to 29% in embryos with higher zona pellucida variation. Palmstierna et al. [93] analysed that zona pellucida thickness variation exhibited high predictive values for pregnancy outcome and was thus considered an important indicator for good embryo quality [91].

Perivitelline Space Abnormalities: PVS Granularity and Large PVS

The PVS of human oocytes may vary in size (enlarged or not) and content (presence or absence of the grain) [35, 42]. It was estimated that oocytes with a large PVS developed worse after ICSI (37.5%) than those with a normal PVS (60.3%) [36].

The nature and origin of PVS granules still remains to be determined, but there is some evidence that this debris may derive either from coronal cell process remnants [94] or from an extracellular matrix [95]. Sathanathan [94] showed that oocytes with granularity in the PVS have an increased risk of developmental incompetence [94], and PVS granularity, considered to be an extracytoplasmic abnormality, may be a sign of gonadotropin overdose, since the percentage of oocytes with perivitelline granules was significantly higher in the high-dose (>45 ampoules) stimulated group compared with the low-dose (<30 ampules) stimulated group [20]. Earlier studies also indicated that in 15% of meiotically mature human oocytes, an incomplete and premature exocytosis of cortical granules can occur [41] and that PVS granularity may be a sign of gonadotropin overdose [20]. Other studies [95, 96] found that extracellular matrix compromising granules and filaments in the PVS are identical to the matrix observed between the cumulus and corona radiate cells. The study by Balaban and Urman [5] commented that PVS granularity may be a physiological maturation-related phenomenon that has no effect on fertilization and cleavage rates, embryo quality and the clinical outcome of assisted reproduction [5].

Early studies described that a large PVS could be related to oocyte over maturity [97]. The studies performed on the effect of large PVS appearance had been limited and controversial. Balaban et al. [42] showed that the appearance of large PVS has no detrimental effect on the fertilization rates, cleavage and embryo quality. In contrast, other studies demonstrated significantly decreased fertilization rates for injected oocytes with large PVS compared to oocytes with normal PVS. The study by Rienzi et al. [55] showed that the presence of large PVS is related to lower fertilization rate and higher incidence of abnormal pronuclear morphology. Besides these parameters, embryo quality was not affected by the appearance of large PVS.

Evaluation of Giant Oocytes

Giant oocytes can rarely be seen after hormonal stimulation for human assisted reproductive technologies. Although oocyte dysmorphism seems to have little effect on the rate of aneuploidy, giant oocytes which are usually diploid are more likely to cause triploidy after fertilization. Oocyte meiosis is very sensitive to endogenous and exogenous factors that may cause the production of oocytes with chromosomal abnormalities and, therefore, of abnormal zygotes. It is presumed that intra- and extrafollicular influences (perifollicular microvasculature, oxygenation, the presence of residues from cigarette smoke) may disturb maturation, leading to giant oocytes with an increased rate of aneuploidy [98].

Giant oocytes have been shown to be 30% larger in diameter than normal oocytes. The study by Balakier et al. [99] examined different morphological patterns amongst giant unfertilized and fertilized oocytes. All unfertilized oocytes appeared to be diploid and contained one or two metaphase plates (46 or 2×23 chromosomes) and one or two polar bodies. Fertilized giant oocytes exhibited either two or three pronuclei or two or four polar bodies. Both types of giant zygotes were reported to be capable of normal cleavage and development to blastocyst stage. However, chromosomal analysis of

the embryos derived from these oocytes showed that they were all abnormal with numerical alterations indicative of ploidy change. Therefore, as these giant oocytes might be a possible source of human digynic triploidy, they should be excluded from transfer to avoid undesired miscarriages [99].

Recently, the study by Ebner et al. [29] also indicated that giant oocytes are produced either by lack of cytokinesis during mitotic division or by fusion of two oogonia and, consequently, are associated with a diploid set of genetic material. These mechanisms explain the binucleate state of immature giant eggs [100]. Since patients who developed giant gametes exhibited significantly higher levels of oestradiol and increased numbers of retrieved oocytes, this abnormality may be linked to ovarian stimulation [99]. The resulting zygotes from giant oocytes will have a triploid chromosomal set after DNA replication and be capable of normal cleavage and adequate blastocyst formation. Ebner et al. [29] also recommended excluding giant oocytes from transfer, even if they carry the regular number of two pronuclei. As a result of the evidence-based data, exclusion of giant oocytes from IVF trials is recommended. In case of fertilization, giant oocytes even with 2PN should be treated like multipronuclear zygotes.

Effect of Number of Morphological Abnormalities on Developmental Competence of the Oocyte

The effect of the number of morphological abnormalities of oocytes on the quality and the implantation potential of derived embryos has not been studied in detail. De Sutter et al. [36] showed that the fertilization rate and embryo quality of the oocytes with no abnormality, one or two or more abnormalities were similar. In a later study, Balaban et al. [42] demonstrated that the incidence of two abnormalities in a group of oocytes with morphological deviations was 26%, whereas this rate decreased to 6% for three abnormalities. Fertilization rate and embryo quality did not differ in the group of oocytes with no abnormality or one, two or three morphological abnormalities. In contrast, Loutradis et al. [58] showed a deleterious effect of the type and the number of only cytoplasmic abnormalities on the developmental potential of the oocyte and reported that the severity and the number of cytoplasmic defects have a deleterious effect on embryo quality. A more recent study on in vitro matured oocytes also showed that embryo quality was decreased in parallel to the number of the morphological abnormalities of the oocyte from which it was derived [97].

Yakin et al. [86] demonstrated that embryos derived from oocytes with multiple morphological abnormalities, especially when at least one single cytoplasmic dysmorphism is included, have a decreased blastocyst formation rate, and once the blastocyst is formed, the quality and hatching status is detrimentally affected. The same study also showed that aneuploidy rate of embryos derived from oocytes with multiple abnormalities including at least one cytoplasmic abnormality is significantly higher than the group of embryos derived from oocytes with single abnormality or normal morphological appearance.

Effect of Oocyte Morphological Abnormalities on the Cryopreservation Outcome

There are many published studies on the effect of abnormalities of oocyte morphology on embryo quality and viability. Whether morphological abnormalities of the oocyte influence cryosurvival and further development of derived embryos is not well known. The study by Balaban et al. [87] compared cryosurvival and progression to the blastocyst stage of 5,292 frozen–thawed embryos derived from normal and abnormal oocytes. The authors showed that the presence of a cytoplasmic abnormality of the oocyte significantly decreased cryosurvival. This detrimental effect was more pronounced in embryos derived from oocytes with vacuolar cytoplasm or with central granulation.

Furthermore, these embryos did not have the potential to develop into good quality blastocysts or reach the hatching stage. On the other hand, presence of a single extracytoplasmic abnormality of the oocyte did not affect cryosurvival and the potential to develop into good quality blastocysts. Grade 2 embryos derived from oocytes with irregular shape or a large PVS had decreased cryosurvival. However, when these embryos survived cryopreservation, their potential to develop into good quality blastocysts or to reach hatching stage was unaffected. Even if clinical outcome of frozen–thawed embryo transfer cycles in relation to oocyte morphology cannot be inferred from the results of this study as multiple embryo transfer was performed, it has been shown that the developmental behaviour of frozen–thawed embryos derived from multiple dysmorphic oocytes was similar when compared to results from the literature reported for fresh embryos [5, 21].

Different hypotheses might be introduced to explain the decreased cryosurvival and blastocyst development rates from morphologically good-looking embryos that are derived from oocytes with severe cytoplasmic defects. One of them is the increased rate of aneuploidy found in the embryos derived from such oocytes [40]. A more recent study by Yakin et al. [86] showed that cytoplasmic and multiple abnormalities where at least one cytoplasmic abnormality was included significantly impaired blastocyst development [86]. Although a 20% higher aneuploidy rate was found amongst embryos derived from these oocytes, the difference did not reach statistical significance.

An alternative hypothesis to explain reduced cryosurvival of embryos derived from oocytes with a non-homogenous cytoplasm may be prevention of dissemination of the cryoprotectant within the cell due to the presence of cytoplasmic vacuoles or centrally located granulation. Proper dissemination of the cryoprotectant within the cell is crucial for cryosurvival. It is important to note that, similar to the behaviour of fresh embryos derived from different types of morphological abnormalities, the presence of extracytoplasmic abnormalities alone does not affect blastocyst development despite decreasing cryosurvival. However, embryos derived from oocytes with vacuolar cytoplasm or central granulation do not seem to bear the potential to develop into good quality blastocysts or to reach the hatching stage after cryopreservation. These cytoplasmic abnormalities may be reflections of genetic, epigenetic or metabolic defects in the oocyte. Embryos with severe cytoplasmic abnormalities comprise around 5% of all embryos suitable for cryopreservation.

Women who have all of their excess embryos derived from oocytes bearing severe cytoplasmic abnormalities should be counselled about the reduced chance of these embryos developing into good quality blastocysts or reaching the hatching stage. It is difficult to assume that transfer of such an embryo will result in pregnancy. Cryopreservation and subsequent transfer of embryos derived from oocytes with severe cytoplasmic abnormalities should be avoided [87].

Genetic Constitution of Oocytes with Morphological Abnormalities

Based on the published data, it appears that the developmental potential of oocytes with severe cytoplasmic defects is significantly impaired. However, it is still not known for certain if these cytoplasmic dysmorphisms are a reflection of a developmental defect in the oocyte or if the dysmorphism itself is inhibitory to the eventual development of the oocyte and subsequent embryos. One of the possible reasons shown is the defective genetic constitution of these oocytes. Van Blerkom and Henry [41] showed that as many as half of the oocytes with dysmorphic phenotypes that arise early in meiotic maturation are aneuploid, with hypohaploidy being predominant. In contrast, cytoplasmic defects which occured at or after metaphase I are associated with a relatively low frequency (<15%) of aneuploidy which is comparable to that reported for oocytes with a normal cytoplasmic appearance [101]. Certain types of cytoplasmic abnormalities such as organelle clustering have also previously been shown to be associated with a high degree of aneuploidy and reduced oocyte and embryo metabolism [40].

Alikani et al. [37] analysed the relationship between variations found in oocyte morphology and aneuploidy as well as abnormal fertilization, embryo development and pregnancy and implantation rates. According to other authors [86], oocyte dysmorphism was not associated with a higher risk of aneuploidy in the developing embryo. Although statistically insignificant, oocytes with cytoplasmic dysmorphism and oocytes showing multiple morphological abnormalities were associated with lower fertilization and cleavage rates, as well as higher risk of aneuploidy in the derived embryos.

Kahraman et al. [44] showed that 53% of the embryos derived from the group of oocytes with centrally granulated cytoplasm were aneuploid. Although the genetic constitution of the oocytes displaying cytoplasmic abnormalities was studied, the fate of the oocytes with extracytoplasmic abnormality is not very clearly examined by earlier studies. A more recent study by Yakin et al. [86] demonstrated that although the aneuploidy rate of the group of embryos derived from oocytes with extracytoplasmic abnormalities (46.7%) was similar to the group of embryos derived from oocytes with normal morphology (41.8%), the aneuploidy rate for the group of embryos derived from oocytes with cytoplasmic abnormality was higher (60.0%) when compared with the group of embryos derived from oocytes with extracytoplasmic abnormality and the group of embryos derived from oocytes with normal morphology [86].

Yakin et al. [86] reported that embryos that developed from oocytes with normal morphology showed a lower (41.9%) aneuploidy rate when compared with embryos that have developed from oocytes with different types of morphological abnormalities in total (51.7%). However, this difference was not statistically different. When embryos were categorized according to the morphological abnormalities of the oocytes which they had been derived from, except the cytoplasmic abnormality group, all the other groups showed similar rates of aneuploidy. Embryos derived from oocytes with cytoplasmic and multiple abnormalities that contained at least one type of cytoplasmic abnormality showed the highest rate of aneuploidy (60.0 and 61.8%, respectively); however, the difference was still not statistically significant related to the low sample size in some study subgroups. As in this study no statistically significant differences for the chromosomal constitution of the normal group and morphological abnormality groups were found, the authors speculated that other defects like gene expression alterations may lead to failure in blastocyst formation. Wells et al. [102] demonstrated an association between certain forms of abnormal oocyte and zygote morphology and disturbances of gene activity.

Changes in oocyte mRNA associated with advancing maternal age have been reported, potentially linked to the well-documented age-related increase in oocyte aneuploidy. The application of the same technology has also revealed that morphologically abnormal preimplantation embryos frequently display atypical patterns of gene expression [103].

These findings suggest that the analysis of gene expression is a worthwhile approach for the identification of new viability markers [104].

It is well known that appropriate gene expression is vital for the regulation of metabolic pathways and key development events. The study by Wells et al. [102] examined the expression of nine genes in human preimplantation embryos and determined whether certain types of oocyte abnormalities such as granular cytoplasm, cytoplasm with condensed organelles or irregular shape are associated with altered gene activity. Altered BUB1 and BRCA1 gene expression was found in the condensed organelles group, whereas BUB1 alteration was observed for granular cytoplasm. No unusual expression of MAD2 was observed for the group of blastocysts derived from oocytes with irregular shape.

Fully validated microarray approaches involving the analysis of whole oocytes will be the near future applications for identifying oocytes destined to produce chromosomally normal, viable embryos with high likelihood for live birth [104].

Conclusions

After oocyte collection for assisted reproductive technologies, the oocytes display various aspects of maturation, integrity and viability. As there is still a lack of reliable and rapid biochemical, or molecular marker of their status, the best modality for evaluation of oocyte quality still remains to be direct microscopic observation of morphology [28].

Morphological assessment of human oocytes retrieved for ART remains to be a major tool in predicting IVF or ICSI outcome and should be further developed [28]. There are no clear and well-defined criteria for oocyte morphology evaluation as there are no widely accepted grading systems in effect. MOMS (metaphase II oocyte morphological scoring) should be introduced into human IVF laboratory practice, especially where oocyte selection before insemination is required. Moreover, universal MOMS could be used to unselect oocytes that have a lower chance to form a viable embryo for insemination (to reduce the creation of supernumerary embryos) as well as for cryopreservation [55].

Despite the fact that clear decisions for all types of morphological differences of human oocytes cannot be based upon data, some recommendations for specific abnormality types can be proposed.

Although results regarding the effect of only extracytoplasmic morphological deviations are still controversial, few studies were able to demonstrate their detrimental effect on the implantation potential of the embryo and further fate of the newborn. Therefore, in line with the published data, these types of oocyte dysmorphisms should perhaps not be considered as abnormalities but only a phenotypic deviation resulting from the heterogeneity of the oocytes retrieved.

In contrast to extracytoplasmic abnormalities, it is very clear that severe cytoplasmic deviations of the oocyte (organelle clustering also mentioned as sERCs, centrally severe granulation and excessive vacuolization) do impair the developmental and implantation potential of the embryo and may have important genetic consequences on the newborn [5, 19, 21, 105].

It can be concluded that morphological evaluation of the oocyte should be coupled with a detailed evaluation of the resulting embryo to reach a valuable conclusion regarding implantation and pregnancy. Certain morphological characteristics indicate a high likelihood of abnormality, and caution should be exercised when transferring embryos derived from such oocytes.

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Polarization Microscopy

14

Markus Montag, Maria Köster, and Hans van der Ven

Abstract

The potential of an embryo to implant is a key element for the success in assisted reproduction. Morphological criteria of oocytes and embryos and developmental progression during the preimplantation phase were considered as a strong predictor for embryo implantation. Recently, polarization light microscopy has enabled the detection of structures within oocytes which possess a natural birefringence. Birefringence analysis of living human oocytes represents a new approach in assessing oocyte viability and embryo potential. The present knowledge of birefringence imaging will be summarized especially in regard to their impact on assisted reproduction.

Keywords

Polarization microscopy • Zona • Spindle • Oocyte • Birefringence

The emerging field of cell biology in the nineteenth century was strongly influenced by advances in light microscopy. However, the use of polarized light at that time was rather low. In the first half of the twentieth century, Schmidt published a first systematic study of living animal cells and tissues and described the structure and development of skeletal and cellular components using polarization microscopy [1]. Later polarization microscopy was applied to study spindle dynamics in living cells [1–3]. Inoué and co-workers were the first to show the relationship between spindle retardance and microtubule density [4]. The introduction of video-enhanced microscopy greatly improved the sensitivity of polarized light microscopy [5].

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Polarization Microscopy in Assisted Reproduction

The first investigation published on the application of polarized light in male gametes dates back to 1875, there Engelmann reported that the frog sperm tail shows birefringence, whereas the sperm head does not [6]. The very first photographic records of the spindle and astral birefringence in sea urchin zygotes were published in 1937 [7].

However, polarization microscopy only entered the field of reproduction in the late twentieth century, when advanced computer technology became available which allowed processing the huge amount of data generated in real-time imaging. The new polarization microscope system used liquid crystals to modulate the polarization state and enabled real-time visualization of birefringent structures [8]. This type of instrument was the first with a proven applicability in assisted reproduction. The first publications on embryological specimens described the two elements within the mammalian oocyte which are birefringent: the zona pellucida [9] and the meiotic spindle [10] (Fig. 14.1a).

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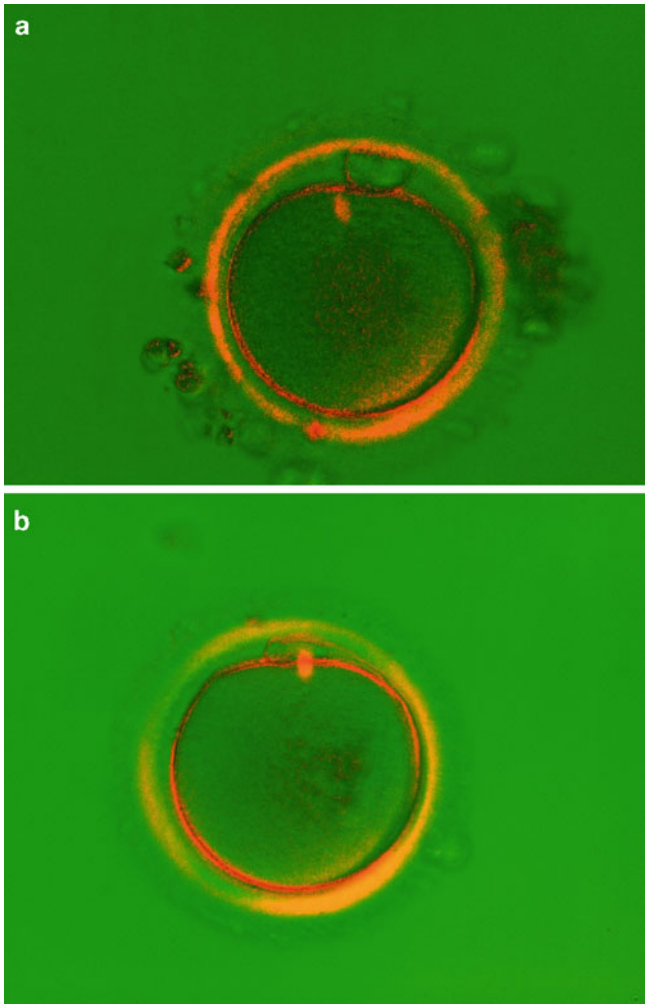

Fig. 14.1 Birefringence in human oocytes. The birefringence of the inner ring of the zona pellucida and the spindle underneath the polar body can be easily detected by polarization microscopy (**a**). In some oocytes, the spindle can be either absent or located at a different position. (**b**) An example of a spindle strand between the first polar body and the oocyte is shown

Spindle Imaging

Spindle Dynamics in the Meiotic Cell Cycle

The spindle is a highly dynamic structure, and especially during the progression of the meiotic cell cycle, the spindle disappears during the transition process in telophase I [11]. More detailed investigations on the course of the meiotic cell cycle were performed in human metaphase-I oocytes derived from stimulated cycles which were matured to metaphase-II in vitro [12]. This study showed that following the extrusion of the first polar body, the spindle formed a connective strand between the first polar body and the ooplasm prior to complete spindle disassembly (Fig. 14.1b). For a considerable time period, no spindle was detectable in the oocytes, followed by formation of the metaphase-II meiotic spindle which appeared underneath the first polar body approximately 115–150 min after extrusion of the first polar body from the oocyte. This study also showed that the presence of a first polar body by conventional light microscopy does not necessarily allow classifying an oocyte as metaphase-II.

Presence and Location of Spindles in Oocytes

As soon as spindle imaging was available in the IVF laboratory, the question arose whether it is relevant to locate the spindle during ICSI or not [10]. It became evident that the first polar body is not a reliable predictor of the location of the metaphase-II spindle. Rienzi et al. [13] reported that oocytes with a deviation of the spindle location from the position of the polar body of more than 90° showed lower fertilization rates, but that spindle position had no effect on embryo development, although another paper reported on the contrary [14]. Nowadays, it is believed that dislocation of the polar body from the spindle position may be a result of manipulation and stress caused during oocyte denudation [15], and therefore, the relevance of this point is unclear at the moment.

The relevance of the presence of the spindle for the outcome of ICSI was investigated in numerous publications with contradictory results in terms of fertilization rates, embryonic developmental competence on day 3, blastocyst formation rates, pregnancy and implantation rates (for references, see [16]).

A recent meta-analysis investigated the influence of the meiotic spindle visualization in human oocytes on the outcomes after ICSI [16]. The authors included ten published trials, although there was heterogeneity among some of the studies. The overall results showed for oocytes with a detectable spindle statistically higher fertilization rates, cleavage rates and embryo developmental rates up to the blastocyst stage. However, there was no benefit in terms of higher pregnancy or implantation rates.

Cryopreserved Oocytes

Polarized light microscopy was used to study the effect of cryopreservation on the spindle in metaphase-II oocytes. Using a conventional slow-freezing/rapid-thawing protocol, spindles reappear within 3 h after the thawing process in more than 50% of the oocytes [11, 17]. Using of a very efficient slow-freezing protocol with optimized sucrose concentrations, a reformation of the spindle was achieved in more than 80% of the frozen-thawed oocytes within 1 h after thawing [18].

Studies investigating the influence of oocyte vitrification on the spindle reported that spindles were found in 50% of the warmed oocytes, and in another 25%, the spindle appeared within the following 2 h [19]. Others reported that during the vitrification process, metaphase-II oocytes spindles remained present and did not disappear [20]. However, a temperature drop below 37°C resulted in spindle depolymerization, whereas maintaining the temperature at a physiological point left the spindle intact and unaffected. These data show that the method of vitrification or slow freezing may have an influence on the spindle dynamics and may differ from lab to lab.

In Vitro Matured Oocytes

Spindle imaging is a good tool to follow the process of in vitro maturation and to decide on the optimal timing for ICSI in in vitro matured oocytes [21]. This is especially important if an in vitro maturation cycle is based on the presence of germinal vesicle (GV) stage oocytes, as oocytes matured from GV stage in vitro are not in synchrony during the following maturation process. Hence, timing of ICSI is of uttermost importance as in vitro matured oocytes do show a different time course compared to oocytes from stimulated cycles and develop faster [21].

Several publications have shown that the spindle of in vitro matured oocytes is very sensitive in regard to temperature [22]. Further, in vitro matured oocytes exhibit a high frequency of chromosome misalignments, probably due to spindle fragrance [23]. Like in oocytes from stimulated cycles, the location of the spindle is also positively correlated to the fertilization rates in in vitro matured oocytes [24].

Spindle Imaging and Laboratory Parameters

Spindle imaging is an ideal tool for quality assessment of certain laboratory parameters. Spindles are sensitive to pH and temperature, and it was shown that human spindles start to disintegrate at a temperature of 33°C. Once disintegrated, spindle reassembly depends on how long it was exposed to the minimal temperature. Spindle reformation is very unlikely if the temperature dropped below 25°C [25].

Exposure of oocytes in culture medium without stabilization of the pH in the medium also causes spindle disassembly within 8–10 min [26]. If spindle reformation after a pH shift is also dependent on a certain threshold is not known at present. Therefore, successful spindle imaging is a criterion that the settings for pH and temperature are correct and that manipulation of oocytes during the procedures preceding spindle imaging, like denudation and ICSI, did not lead to relevant changes.

Zona Imaging

Zona Birefringence and Zona Architecture

In conventional light microscopy, the zona pellucida of mammalian oocytes appears as a uniform layer surrounding the oocyte. However, polarization microscopy of hamster oocytes revealed a multi-layer architecture where three layers within the zona pellucida can be distinguished by their birefringent properties [9]. The inner zona layer exhibits the highest amount of birefringence, followed by a thin middle layer devoid of birefringence and an outer layer with a faint birefringence. The same characteristic pattern was also found in the zona pellucida of human oocytes [27]. How this relates to the known components of the zona pellucida, the zona proteins (ZP) and the embedded glycoproteins and polysaccharides is still unknown. It is commonly believed that the extent of birefringence of the inner zona layer is primarily an indication for the degree of order of the contributing structures within the zona during oocyte maturation.

Zona Imaging as a Prognostic Factor

Assessment of the zona pellucida by conventional microscopy and without the information of polarization microscopy cannot be used as a predictive factor for the success of ICSI [28]. However, Shen et al. found variations in the birefringence intensity of the inner layer of the zona pellucida among different oocytes by measuring zona thickness and intensity at three different positions of the entire zona. A further retrospective analysis showed that the mean zona birefringence intensity and thickness of the inner zona layer were higher among conception vs. non-conception cycles [29]. Using the same measuring approach like Shen et al., Rama Raju reported from retrospective data a correlation between zona birefringence and the potential of an embryo to develop to the blastocyst stage [30]. These studies stimulated further prospective investigations on the potential of zona birefringence imaging as a prognostic factor in ART.

Embryo Development and Pregnancy Outcome

One study investigated the intensity and uniformity of the zona inner layer's retardance in unfertilized metaphase-II oocytes by a non-invasive single observation prior to ICSI treatment [31]. Based on zona birefringence as the only selection criterion, two fertilized oocytes were selected for further culture and transfer. In this prospective study, implantation, pregnancy and live birth rates were significantly higher in cycles where the transferred embryos were

derived from oocytes with high birefringence compared to those involving oocytes with low birefringence. Furthermore, embryo development on day 3 but not on day 2 was superior in embryos derived from high birefringent oocytes compared to embryos from low birefringent oocytes.

Meanwhile, two different approaches for automatic sampling of measurement values and automatic zona imaging have been presented [26, 32]. The first approach is based on the analysis of the radial orientation of glycoproteins in the inner zona layer [32]. The angular deviation of the radial orientated structures is greater if the inner zona layer is disrupted or less uniform and hence a characteristic for a presumably suboptimal oocyte. Data from a prospective clinical study supporting this theory are not yet available.

The second approach uses a software module which automatically detects the inner birefringent zona layer [26]. Following detection, a zona score is calculated in real-time based on the intensity and distribution of the birefringence at 180 measuring points. This enables an objective and user-independent score of the corresponding oocyte within a short observation time. It was shown that the results of automatic zona imaging were comparable to the data from the subjective study mentioned above [31].

In a prospective study, Ebner et al. used automatic zona imaging at the oocyte stage prior to ICSI and cultured embryos up to the blastocyst stage. When the automatic detection of the birefringence of the inner zona layer in the oocytes failed due to a heterogeneous intensity and thickness of the inner zona layer, the corresponding embryos showed significantly lower compaction rates and blastocyst formation rates. In addition, these embryos were significantly less involved in the initiation of a pregnancy. They concluded that the automatic zona score was a strong predictor of blastocyst formation rate [33]. In another prospective study, a positive correlation between zona pellucida birefringence score and implantation and pregnancy rates was reported [34–36]. This study showed for the first time that the miscarriage rate was higher in embryo transfer cycles where the transferred embryos were exclusively derived from oocytes with a low zona birefringence score.

Most of the studies conducted so far show that oocyte zona birefringence is a good predictive criterion for embryo implantation potential. Zona birefringence probably reflects the structural integrity of the zona pellucida. Oocytes with a high zona birefringence possess a very regular and optimal structured zona which is an indication for a good follicular development. Therefore, these oocytes may also have an optimal cytoplasmic potential which favors a good developmental competence for embryonic growth and implantation. Preliminary data indicate that different zona birefringent patterns (Fig. 14.2) correlate with different expression profiles of certain candidate genes in subpopulations of the cumulus-oophorus complex [35].

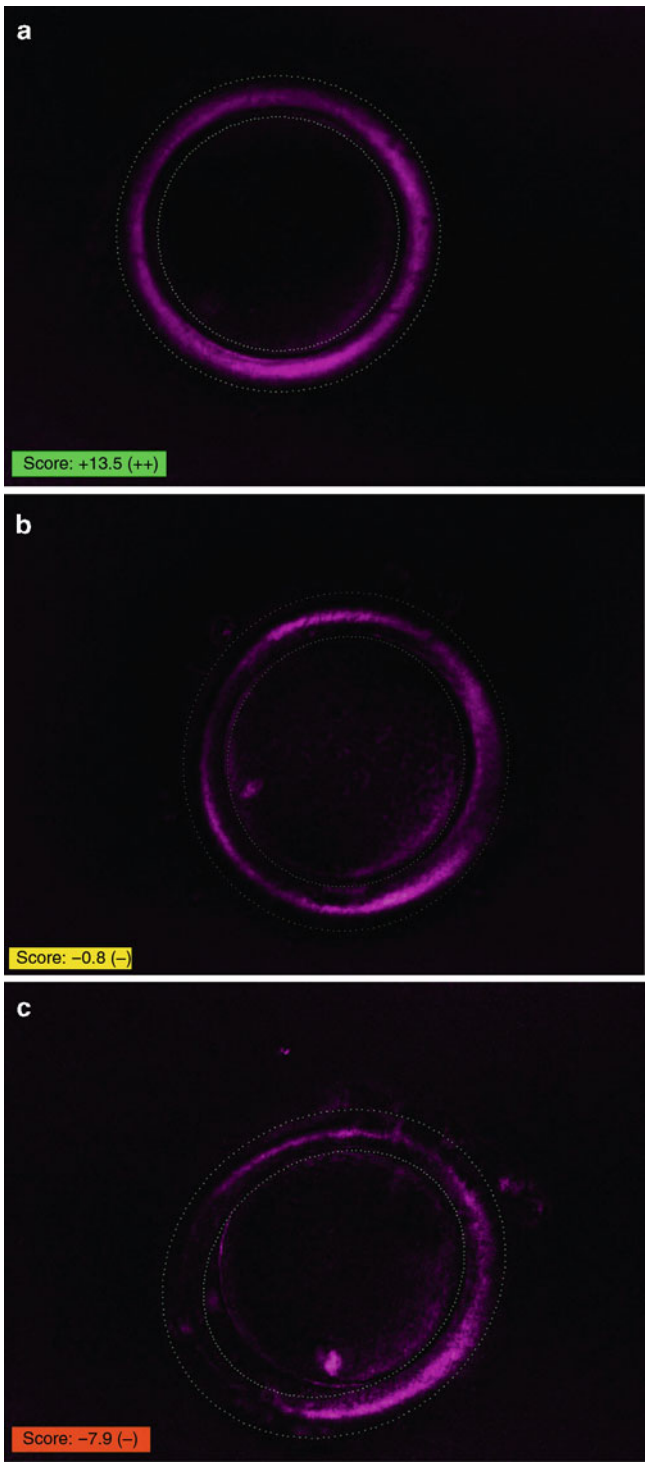

Fig. 14.2 Zona imaging by polarization microscopy. The different patterns of the birefringence of the inner zona layer can be automatically assessed and are characteristic for oocytes with a good (**a**), intermediate (**b**) or poor (**c**) embryo implantation potential

Summary

Polarization microscopy is on its way to become an important add-on in the laboratory for assessing the competence of gametes in assisted reproduction. The availability of polarization

microscopes which can be used in the routine daily laboratory work is granted, and the use of this technique as a new tool in characterizing the developmental potential of oocytes could be proven by now in numerous studies. The applicability of polarization microscopy for classifying spermatozoa has been shown in some initial studies, too [36, 37], and this is an open field for further improvements. However, until to date most studies performed on the basis of polarization microscopy are more or less descriptive and/or comparative. What is really needed is the knowledge of the underlying physiological processes which result in the phenomenon which we describe. This may lead to improve laboratory practice as well as optimizing or individualizing stimulation protocols.

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Cumulus Cell Gene Expression in Assessment of Oocyte Quality

15

Dagan Wells

Abstract

Cumulus cells originate from granulosa cells and surround oocytes from the time of follicular antrum formation until after fertilization. The cumulus cells have essential functions in the ovary, mediating transmission of endocrine signals and supporting oocyte growth and maturation. The relationship between oocytes and their associated cumulus cells is extremely intimate. Cytoplasmic projections extend from the innermost layer of cumulus cells, penetrating the zona pellucida and forming gap junctions at the oocyte surface. This allows for direct exchange of macromolecules, a bidirectional communication essential for the production of competent oocytes. The fact that cumulus cells are so closely associated with the oocyte, and share the same microenvironment within the ovary, has led to suggestions that information concerning oocyte quality might be obtained by analyzing them. Several studies focused on cumulus cell gene expression have now been published, indicating that a noninvasive assessment of oocyte potential, based upon analysis of the surrounding cumulus cells, may indeed be possible.

Keywords

Human • Oocyte • Cumulus cells • Transcriptome • Gene expression

Commentary

Cumulus Cell Biology and the Cumulus–Oocyte Relationship

The mature cumulus–oocyte complex (COC) is composed of the secondary oocyte, arrested at metaphase II following extrusion of the first polar body (PB), and surrounding cumulus cells (CCs). A unique characteristic of CCs is the presence of highly specialized cytoplasmic projections that pierce through the zona pellucida and form gap junctions at their tips with the oocyte [1]. This intimate association allows CCs to fulfill vital roles, supporting the maturation of the oocyte and relaying endocrine and other environmental signals.

Cumulus cells (CCs) originate from granulosa cells, the primary type of somatic cell in the follicle. Initiation of GC differentiation occurs upon follicular antrum formation, corresponding approximately to the end of the oocyte growth phase. In humans and other mammals, two anatomically and functionally distinct lineages are generated—mural GCs that line the wall of the follicle with primarily a steroidogenic role and the CCs, which encircle the oocyte [2, 3].

Cumulus cells undergo extensive proliferation prior to LH surge, and following the preovulatory LH surge, a cascade of events is initiated that leads to further proliferation and expansion [4, 5]. The competence to undergo expansion is a unique characteristic of CC differentiation [6], which has been shown to be critical for normal oocyte development, ovulation, and fertilization [7–9].

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CCs are known to metabolize the bulk of glucose consumed by the COC, supplying metabolic intermediates like pyruvate, mainly via glycolysis, to the oocyte (for a detailed review on role of glucose metabolism in the oocyte, refer to Sutton-McDowall [10]). Other substrates of low molecular weight such as amino acids and nucleotides are passed to the growing oocyte for its own synthesis of macromolecules as well as ribosomal and messenger RNA from the GCs/CCs.

The nutritional support, trafficking of macromolecules, and dissemination of endocrine signals that this system allows may be particularly important for oocytes due to the avascular nature of the granulosa layer (reviewed by Johnson and Albertini [1, 11]). This communication is so crucial that genetic deletion of the oocyte specific gap junctional subunit, connexin-37, leads to female sterility in mice, resulting from a lack of mature follicles, failure to ovulate, and development of numerous inappropriate corpora lutea.

The role of CCs in supporting in vivo oocyte development as well as IVM [12, 13] has led increasing numbers of researchers to study these cells. Not only do investigations in this area promise to shed light on the biology of the follicle and the mechanisms promoting oocyte competence, but it is also possible that new biomarkers of oocyte potential may be identified.

One important component of the follicular environment, relevant to oocyte viability, is oxidative stress. Antioxidants produced by CCs, such as superoxide dismutases (SOD), are postulated to protect the oocyte from damage caused by reactive oxygen species. SOD levels in CCs have been noted to decrease with advancing female age, and higher SOD activities were associated with successful outcomes in assisted reproduction techniques [14]. Glutathione S transferases are another class of enzymes known to protect cells from reactive oxygen species. In a study by Ito et al. [15], GSTT1 (glutathione S transferase theta 1) was shown to be a good indicator of age-related infertility. Not only does this data emphasize the influence of oxidative stress on oocyte viability, it also suggests that SOD and GSTT1 might serve as potential biomarkers of prognostic significance.

Other studies have indicated that apoptosis rates are elevated for CCs associated with morphologically abnormal oocytes [16]. An increase in CC apoptosis has also been associated with immaturity of human oocytes, impaired fertilization [17], suboptimal blastocyst development [18], and poor IVF outcomes [19, 20]. It may be that abnormal/poor-quality oocytes induce apoptosis in the associated CCs. Alternatively, CCs with high levels of apoptosis, perhaps symptomatic of a suboptimal follicular environment, may lead to impaired oocyte development. Whatever the explanation, these observations highlight the interdependence of the oocyte and its CCs and suggest that certain elements of CC biology may serve as indicators of oocyte viability.

Gene Expression Studies of Human Cumulus Cells: Identifying New Biomarkers of Oocyte Quality

With appropriate methods, it may be possible to detect and decode molecular alterations in the CCs associated with differences in oocyte viability [21]. For example, patterns of gene expression reflect processes occurring within a cell at a given moment in time, including the cell's responses to environmental challenges. Thus, patterns of gene activity in CCs may reveal much concerning the conditions within the follicle during the final stages of oocyte maturation. Several groups have utilized emerging transcriptomic techniques to gain a better understanding of follicle biology and to try and identify novel biomarkers of oocyte competence [22–29] (see Glossary).

CCs are constantly responding to the intrafollicular environment to ensure optimal oocyte development, adjusting gene expression in order to maximize oocyte support and minimize damage caused by extrinsic factors (e.g., reactive oxygen species). An ongoing study of the CC transcriptome in our laboratory has indicated that the follicular microenvironment might even play a role in the origin of oocyte meiotic chromosome abnormality, one of the main causes of oocyte incompetence. The study revealed that cumulus cells associated with aneuploid oocytes have characteristic deviations in their gene expression profile [29] (Fragouli, Wells, and Patrizio, unpublished data). Abnormally expressed genes include several involved in pathways related to cellular stress (e.g., hypoxia, nutritional deprivation), suggesting an association between aneuploidy and suboptimal environment. Some genes involved in hormonal response also displayed abnormal expression, potentially providing a link between the increased frequency of aneuploidy and the altered hormone levels seen with advancing age. Furthermore, a number of genes with roles in apoptotic pathways showed distorted expression levels, in keeping with previous studies suggesting an association between CC proliferation and/or apoptosis and poor IVF outcomes [17–19, 30].

Gasca and colleagues [23] attempted to identify potential regulators and marker genes involved in oocyte maturation by screening human oocytes and CCs using microarrays [23]. Their study identified a number of potentially significant genes involved in processes such as cell cycle checkpoints and DNA repair, including *BARD1, RBL2, RBBP7, BUB3* and *BUB1B*. Appropriate expression of these genes may have relevance to oocyte quality, although this remains to be conclusively proven.

Another microarray study, conducted by Assou et al. reported patterns of CC expression associated with embryo morphology and pregnancy outcome. These included upregulation of *BCL2L11* (involved in apoptosis) and *PCK1*

(involved in gluconeogenesis) and downregulation of *NFIB* (a transcription factor). The researchers proposed that these three genes might be useful biomarkers for the prediction of pregnancy [28].

Feuerstein et al. [25] assessed the expression of six genes chosen because their expression is induced by the LH peak (*STAR, COX2* and *AREG*) or because of known roles in oocyte lipid metabolism (*SCD1* and *SCD5*) or in gap junctions (*Cx43*). With the exception of *Cx43*, all of the genes displayed increased expression in CCs after resumption of meiosis. Nuclear maturation of the oocyte was associated with increased expression of *STAR, COX2, AREG, SCD1* and *SCD5* in CCs. Interestingly, mRNA transcript levels of these genes were lower and distributed over a narrower range in CCs enclosing oocytes, achieving blastocyst development at day 5/6 than in CCs enclosing oocytes unable to develop beyond the embryo stage.

Further potential markers of oocyte competence identified by gene expression studies include *PTGS2* (prostaglandin-endoperoxide synthase; cyclooxygenase), *HAS2* (hyaluronic acid synthase 2), and *GREM1* (gremlin 1) [31]. CCs associated with oocytes that produced high-quality cleavage-stage embryos were found to have greater numbers of transcripts compared to CCs from oocytes that produced poor-quality embryos (expression of *PTGS2* and *HAS2* was sixfold higher; *GREM1* was 15-fold higher). Complementary results were obtained for *GREM1* and *HAS2* by Cillo and colleagues, suggesting that the measurement of transcripts from these genes in CCs might complement morphological evaluation and provide a useful tool for selecting oocytes with greater chances of fertilization and development in vitro [24].

Altered expression of several genes has been correlated with early cleavage postfertilization, a feature generally considered to be a positive indicator of IVF outcome. The function of these genes suggest a role for hypoxic conditions (*CXCR4, GPX3, DVL3, HSPB1*) or delayed oocyte maturation (*CCND2, TRIM28, DHCR7, CTNND1*) in non-early cleavage embryos [26]. Not only do these results shed light on aspects of follicle biology that might predispose to late/early cleavage, but as with the studies discussed above, they also provide a set of markers that might assist oocyte selection.

A further set of markers of oocyte/follicle competence were reported by Hamel et al. [27] who performed experiments with the aim of identifying cumulus/granulosa cell genes specifically expressed in follicles that produced a pregnancy. They created a DNA microarray composed of cumulus/granulosa cell expressed sequence tags from subtracted libraries (cumulus/granulosa cells from women who became pregnant versus cells from those who did not). Altered expression of the *CDC42, 3bHSD, SERPINE2, FDX1* and *CYPA191* genes was significantly associated with competent follicles that resulted in pregnancies. These correlations were confirmed with quantitative PCR analyses [27].

Conclusion

The various studies seeking to characterize the CC transcriptome have yielded a wealth of novel data, including a detailed catalog of the genes expressed in CCs. Importantly, a number of genes displaying differential activity, apparently related to oocyte competence, have been identified. Quantification of the mRNA transcripts from such genes, or the proteins they produce, may provide new insights into oocyte (and embryo) competence, not possible using conventional techniques. Clinical trials aimed at assessing the potential of CC-based strategies of oocyte quality assessment are now underway. In the near future, diagnostic approaches based upon analyses of cumulus cells may revolutionize the way in which oocytes and embryos are selected for uterine transfer during IVF treatments, potentially leading to increases in fertilization, implantation, and clinical pregnancy rates. If markers of aneuploidy can also be identified, as initial data suggests, a reduction in the rates of miscarriage and aneuploid syndromes (e.g., Down syndrome) are also anticipated. A noninvasive preconception test for aneuploidy would overcome some of the most important technical and ethical difficulties facing preimplantation genetic screening [29].

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Glossary

Downregulated/underexpressed Cases where fewer mRNA transcripts are found. Gene expression is reduced (i.e., the gene is less active).

Gene expression A complete set of all of the genes (i.e., the entire genome) is present in all cells. However, only a fraction of these genes are active in a cell at any given moment. Genes which are being actively transcribed, producing mRNA and ultimately proteins, are said to be "expressed."

Microarray A method for simultaneously quantifying the number of transcripts from large numbers of genes (typically thousands or tens of thousands of genes simultaneously assessed).

mRNA transcripts The molecules that serve as intermediates between genes (made of deoxyribonucleic acid, DNA) and the proteins they produce. The DNA sequence of a gene is transcribed into a messenger RNA (ribonucleic acid) copy, which is subsequently translated into a polypeptide.

Real-time PCR A method of quantifying the number of mRNA transcripts from individual genes. Real-time PCR

is generally considered the most accurate method for quantifying gene expression but only allows analysis of small numbers of genes at a time.

Transcriptome The sum total of all mRNA transcripts found within an individual cell or tissue. The characterization of the transcriptome reveals all of the genes expressed (i.e., active).

Upregulated/overexpressed When two different samples are compared, some genes may be found to have differences in the number of mRNA transcripts. If a sample contains a greater number of mRNA transcripts than expected, the gene is said to be upregulated or overexpressed (i.e., it is more active).

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Part IV

Embryo Culture Methods

Short Culture: Day 1/Day 2/Day 3 Embryo Culture

16

Patrick Quinn

Abstract

I often feel that we have come a long way since the beginnings of clinical human IVF in the early 1980s, but I know that there are many continuing, exciting discoveries yet to be made. In this chapter, after a historical introduction, aspects of in vitro culture during the first 3 days of development will be discussed in the context of the known metabolism and physiology of the embryo. The object will be to meld culture media and the way they are used with the production of early embryos that have the best potential for continued development into a live, healthy baby. I trust that this format and the information herein will therefore contribute to a basis for the continued work on this essential aspect of human IVF.

Keywords

Embryo • Culture media • Embryo transfer • Metabolism • Morphology

Louise Brown, the first successful case of human IVF, was transferred to her mother's uterus as an 8-cell embryo [1]. Normally, this early mammalian preimplantation stage resides in the fallopian tube, and when such embryos are placed in the uterus they perish or are expelled from the uterus [2, 3]. Humans appear to be the only primates in which transfer of early precompaction embryos to the uterus is successful [4]. Soon after IVF became a regular clinical procedure, successful transfer of gametes to the fallopian tube by laparoscopy (gamete intrafallopian transfer—GIFT) was reported [5], and it was suggested that the placement of these cells, and in subsequent reports, the zygote (zygote intrafallopian transfer—ZIFT) [6] and early embryo (tubal embryo transfer—TET) [7], in their correct location, the fallopian tube, was a major reason for the higher success rates of these procedures compared to IVF. However, whereas pregnancy rates of 20–25% for GIFT remained relatively constant over the decade of the 1990s, the success rates of IVF and ICSI with uterine placement of early embryos gradually improved from approximately 10% to a similar 20–25% [8]. This was proposed to be partly caused by the gradual improvement in embryo culture, primarily improvements in embryo culture media rather than any other major parameters such as better ovarian stimulation protocols or transfer of embryos at the blastocyst stage on D5 to their normal site of residence, the uterus [8]. In fact, ongoing pregnancies were also obtained by transferring the gametes directly to the uterus rather than the fallopian tube [9, 10], and although this has never become a routine clinical procedure, it does indicate the plasticity of human gametes and preimplantation embryos with respect to their successful development at various sites in the female reproductive tract albeit their replacement at the site where they would normally reside probably gives optimal results. Embryonic plasticity is also reflected, in my opinion, by embryonic development in vitro in various culture systems, and it is the purpose of this chapter to focus on the factors in such culture systems that influence human embryos during Day (D)1 to D3 of development. The main focus will be on practical aspects of culture of this early developmental stage with some comments related to physiological aspects of the embryo.

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Historical Perspectives of Human Embryo Culture and ET

As mentioned earlier, Louise Brown was transferred on D3 as an 8-cell embryo to her mother's uterus [1]. Trounson et al. [11] reported that embryo transfers (ETs) on D2 at the 2- to 4-cell stage produced the majority of their pregnancies, and this practice was utilized in most IVF programs worldwide in the 1980s. In some countries, D2 ET has remained a popular protocol [12, 13]. It was not until the 1990s that transfers on D3 of embryonic development became de rigueur [14, 15]. The primary reason suggested for this increased pregnancy rate with D3 ET was the ability to select better quality embryos with greater implantation potential on D3 compared to D2 and the elimination of embryos that would have arrested in development between D2 and D3 after transfer on D2 [14]. In addition, activation of the embryonic genome that occurs around D3 of development of the human embryo between the 4- and 8-cell stage [16] has been proposed as another reason for improved selection of embryos from the 8-cell stage onward [17]. Nevertheless, there have been conflicting reports concerning the superiority of ET on D2 or D3 [18, 19]. Subsequently, a similar change in ET to D5 was implemented [20]. Again, the benefits observed of later ET were based on prolonging culture, using it as a tool to select better quality embryos and cull out those that, for genetic or other physiological reasons, had a poorer prognosis for development, and the transfer of the blastocyst embryo to the uterus, the site in which it naturally resides in vivo [20]. In the initial period of D5 ET, some debate arose concerning the superiority of D5 over D3 ET [21, 22]. Some of this disputation may have been due to poor quality embryo culture medium and/or culture technique.

ET on D1 has also been used [23]. The use of this protocol was advocated in situations where adverse culture conditions were having a negative impact on success rates, and so, the early replacement of embryos into the female reproductive tract was a better environmental option for embryos than culture in vitro [24]. It has also been shown that when there is only a small number of oocytes or embryos (5 or less), higher pregnancy rates are obtained with ET on D2 rather than D3, and again, the earlier transfer of embryos to a more congenial environment in the uterus compared to that in culture has been implicated as the prime benefit of this strategy [25, 26].

All of the forgoing observations of the ability of the human oocyte and embryo to produce ongoing pregnancies and live births after transfer on various days of development to various sites in the female reproductive tract indicate the varied capacity of human embryos to fulfill their destiny to develop into a new individual. The remainder of this chapter is focused on culture techniques that participate in this process for the early human embryo.

Metabolism and Physiology of the Early Human Embryo

Energy Substrates

This has been one of the earliest subjects studied and discussed in relation to the development of preimplantation mammalian embryos. The three major substrates are pyruvate, glucose, and lactate, and the general consensus has been to supply these compounds at levels found in the fallopian tube [27]. Despite the fact that there is some debate concerning the acquisition of an accurate estimation of the concentration of these substrates [28], it has been found that low to moderate levels of all three compounds seems to work well with human embryos. The presence of pyruvate is particularly important because, as well as being an essential energy source, it also acts as a sink for removing embryotoxic level ammonium by transamination to alanine; this is one of the reasons why I have excluded alanine from my current series of media for human embryo culture [29]. Low to no levels of glucose also seems to be sufficient for the early human embryos [29]. With respect to lactate, I have combined the provision of this substrate and that of calcium in the form of calcium L-lactate, thus providing the bioactive form of lactate and avoiding excessive chemical levels of lactate by eliminating its D-isomer. The significant effect of additional D-lactate on internal pH (pHi) in the embryo that influences cellular homeostasis has led others to recommend the elimination of D-lactate from IVF media [30].

Amino Acids

Currently, nearly all media for human embryo culture contain amino acids for all stages of preimplantation development, and this is logical as these compounds are present in reproductive tract fluids [31]. Ham's F10, a medium that contains amino acids, was used in the early work of Steptoe and Edwards to culture human embryos [32]. However, in the early 1980s, the use of media such as Tyrode's T6 and my HTF medium [33] that were devoid of amino acids became popular. Subsequently, the use of amino acids became more refined by Gardner et al. [34]. when he proposed the use of nonessential amino acids in the precompaction phase from D1 to D3 of development and then the subsequent addition of essential amino acids and vitamins during the postcompaction period from D3 to D5/6. Important changes to this general theme have ensued. Studies of the consumption and production of amino acids during embryo culture have shown that alanine accumulates in the culture medium indicating its production by the embryo at all stages of preimplantation development (hence, my omission of alanine from my culture

media [29]), whereas leucine is consumed by the early human preimplantation embryos, an obvious reason why this amino acid should be present during culture from D1 to D3 [35]. There has also been a call for the addition of methionine to the cleavage stage medium to lessen the frequency of monozygotic twinning [36], but this relationship needs further study.

It has been known for some time that glutamine readily deaminates, primarily by spontaneous breakdown and secondarily by embryonic metabolism to ammonium, the level of which can become toxic. The initial way to overcome this problem was to change the medium every 48 h to minimize the accumulation of ammonium. As an alternative, I substituted alanyl-glutamine, a stable form of glutamine, for both alanine and glutamine. This was done on the suggestion of Kenneth Drury and had previously been used in media for cell line culture. Subsequently, it was shown that the accumulation of ammonium in embryo culture medium was considerably reduced when using alanyl-glutamine compared to glutamine [37].

Finally, it must also be remembered that amino acids have other roles apart from being the building blocks of proteins and these include acting as energy sources after transamination and acting as internal buffers [30].

Inorganic Ions

The first successful media for preimplantation mammalian embryos were those of Whitten [38] and Brinster [39] and where based on the ionic composition of Krebs-Ringer solution. Some of the more recent changes in ionic composition that have occurred in media for preimplantation human embryos have been the reduction and/or elimination of phosphate ions [40, 41] and calcium magnesium ratios. Increased levels of exogenous magnesium ions in medium reduced the uptake of external calcium by the embryo and thus ameliorated the deleterious effects of excessive calcium within the embryo [42]. We have incorporated this change in magnesium levels in our media based on this work. In my studies with HTF medium [33], I found that excessive ratios of sodium to potassium inhibited mouse embryo development in vitro so we have been mindful of this in our human embryo media and kept the sodium to potassium ratio between 26 and 28 by adding 5 mEq/L of potassium ions. The presence of trace elements in culture media for human preimplantation embryos has not been shown to be of much importance.

pH and Temperature

One of the more important aspects of culture media, in my opinion, is the pH of the medium. Again, using the imitative principle and based on my experience when poor human embryo development was primarily associated with poor pH control [24], I have been an advocate for lower pH levels than the perceived golden role of cell culture which was 7.4 when human embryo culture first began. As far back as 1999, it was suggested that the development of early human embryos was improved using a pH in the range of 7.2 [43]. It was also reported that the internal pH (pHi) of early cleaving human embryos was about 7.12 ± 0.008 [44]. Based on these data, I have recommended a pH of 7.2 ± 0.1 for medium used to culture the early cleaving human embryo from D1 to D3 of culture. Others have recommended a similar value [30, 45]. Most laboratories that have lowered their pH to this range have anecdotally reported improvement in the development of human embryos at this stage. It is now well understood that it is more important to measure pH of the media under culture conditions rather than to just measure the CO_2 concentration in the culture atmosphere [45]. If the pH levels drift out of the recommended range for whatever reason, then CO_2 levels need adjustment to bring the pH back into range [45]. Factors affecting pH levels include altitude, temperature, and the capacity of an incubator to maintain CO_2 levels that can be affected by the frequency of opening the incubator and by leaks in the gas system from its source to within the incubator. It is also good practice to pass the gas mixture through a filter consisting of activated carbon and a HEPE filter to remove volatile organic compounds and particulate matter from the gas. Humidification of the gas is not required if all culture is done under an oil overlay.

In parallel with pH, the temperature at which gametes and embryos are handled and cultured has a critical influence on their subsequent normality and development. First, the actual optimal temperature needs to be determined for human embryos. It has been generally assumed that this is 37°C, but there is some indication that the range may be slightly lower than this, in the range of 36.5–36.9°C [46, 47]. In any event, a sustained effort is needed to keep the embryo within the required temperature limits at all times during development, and probably, the less handling of the embryo to observe the rate of the development, the better [48]. Here is where the use of time-lapse photography taken from within the incubator may play a beneficial role if indeed there are critical events that do occur in the embryo and can be observed during development without disturbing the culture system by removing it from the incubator.

Osmolality

The final aspect of culture medium to be considered is the concentration of dissolved solutes. This is referred to as osmolality. Isotonic saline, a solution of sodium chloride in water, is a fluid in which human erythrocytes neither swell nor shrink [49]. In other words, the concentration of dissolved

sodium chloride in the saline solution is equal to that of dissolved solutes within the cell. Early culture media for human embryos were formulated to have an osmolarity of around 280 ± 5 mosmol/kg of water [50], but it has subsequently been shown that the human embryo can develop in media with a broader range of osmolality, for example, down to 265 ± 8 mosmol/kg. This broader range from 260 to 285 is also similar to that of mouse preimplantation embryos [51].

Formulation of Culture Media

Which Media to Use

Two general strategies, reviewed by Biggers and Summers [28], have been used in the creation of media for human embryo culture: "back to nature" and "let the embryo choose." These two strategies have subsequently come to be associated with a sequential series of media for "back to nature" and a single medium used in a nonrenewed or renewed sequence for "let the embryo choose." In the "back to nature" strategy, which I refer to as the "imitative principle" [29] media components and their concentration are chosen to mimic the chemical composition of that portion of the female reproductive tract where the embryo would be located. In humans, this would be the fallopian tube for D1–D3 and the uterine cavity for D3–D5/6. My fertilization and cleavage media used for insemination in vitro and then culture of the embryo from D1 to D3 are a very successful example of this strategy. These types of media are mainly based on the composition of human tubal fluid, but allowances are made for situations that are known to exist in other mammalian species, primarily the mouse, and studies that have shown that the presence or absence of certain substances and their concentration are of benefit. A prime example of this is the omission of alanine from my media because the embryo excretes alanine following the transamination of pyruvate, and exogenous alanine would inhibit the removal of ammonium by this reaction [29]. Media based on the "let the embryo choose" strategy have focused primarily on the amino acids in the formulation [28]. A range of amino acids that are known to be in the reproductive tract fluids are present in the medium, and it is considered that the embryo can choose which to consume or release and in what concentration [35].

It is likely that further studies, similar to a recent one that showed no difference in outcomes using a single medium for continuous culture from the zygote to the blastocyst stage compared to a sequential media series [52], are required to determine which media series used in which format (renewed vs. nonrenewed) will be the most effective in ART.

Formulation Strategies

This is a simple process providing several simple specifications are followed. It has been described in detail elsewhere [53]. All chemical components, water and vessels, pipettes, containers, etc. (glass and/or plastic) must be as pure as possible, be stored according to manufacturers' specifications, and be prescreened with a mouse embryo assay and/or sperm motility assay and tested for endotoxin levels as appropriate. Even so, there are certain procedures that need to be followed and which I have found will provide a better product. For example, it is mostly recommended that sodium pyruvate be stored at 2–8°C, but Wales and Whittingham [54] found that the stability of this compound was improved, and embryotoxicity was reduced when the solid was stored at or below −40°C. The embryotoxicity was thought to be due to the conversion of pyruvate to the metabolic inhibitor, parapyruvate. Therefore, solid sodium pyruvate should be stored at at least −20°C and not stored in the refrigerator as advised by most manufacturers.

Another practical procedure is to reduce endotoxin levels in solid sodium chloride by heating it at >200°C for at least 1 h. This depyrogenation procedure has no detrimental effect on the properties of the compound.

Amino acids are another category of components that require special attention. Because most amino acids are amphoteric, i.e., they have both acidic and basic groups, they dissolve easier at higher or lower pHs. Therefore, when making concentrated (e.g., 100×) stock solutions, one needs to add acid to get solubilization. For example, to make a 100× stock of Eagle's essential amino acids, add 30 mL of concentrated hydrochloric acid per liter of water before adding the individual components. When making the final medium solution, add components individually and stir continuously until dissolved before adding the next component. A search of the Aldrich-Sigma website (www.sigma-aldrich.com) lists the solvent used to test the solubility of various compounds, including amino acids.

To make medium, the procedure described in Tables 16.1 and 16.2 can be followed. The concentration of various components can be altered by changing the volume of the stock added and/or by changing the concentration of the component in a particular stock. As a precaution, it is advisable to test the efficacy of a particular medium with mouse embryos before using the medium with humans or other species. The method described in Tables 16.1 and 16.2 can also be used to create media to QC the various components of the medium using an appropriate bioassay such as the mouse embryo assay and/or the human sperm motility assay.

Table 16.1 Preparation of media from stocks

Stock	Component	Source and lot #	Amount required	Actual amount
A Stability: 3 months, 2–8°C, filtered	1. NaCl 2. KCl 3. $MgSO_4.7H_2O$ 4. Gentamycin		Add appropriate amounts in grams (g) to 50 mL of water, as indicated in step 5 below 0.005 g ÷ potency, for example, 0.005 ÷ 0.63 = 0.008 g	
Other				
	5. Water		50.0 mL	
Note: Add components 1–4 to a 50-mL Falcon flask, then add the water				
B	1. $NaHCO_3$ 2. Phenol red		0.752 g/50 mL 0.0015 g	
Add $NaHCO_3$ and phenol red to the same 50 mL of water				
C	Na pyruvate		0.036 g/10 mL[a]	
D	Ca lactate pentahydrate		0.0629 g/10 mL	
E	1a. HEPES-free acid[a] or 1b. HEPES Na salt[b] 2. Phenol red		2.979 g/50 mL or 3.254 g/50 mL 0.0015 g	
Adjust to pH 7.35 with (1a.) 1N NaOH or (1b.) 1N HCl before making up final volume to 50 mL. Add these ingredients to 40 mL H_2O. Adjust pH and make up to final volume of 50 mL Alternatively, mix together 1.560 g of HEPES-free acid and 1.550 g of HEPES Na salt into 50 mL of water. When 0.84 mL of this stock is added to a final volume of 10 mL of medium, as described in Table 2, it will give a 21 mM solution of HEPES with a pH of about 7.35 at 37°C				
F Stability: 3 months, −20°C	Stock solutions of amino acids		Make 100× stock in water containing 8.5 g NaCl/L; add HCl as required for dissolution	
G	EDTA Na_4 Make up several days before required; may need to hold at 37°C for several hours to get complete solution		0.004 g/10 mL[a]	
H	Glucose		0.500 g/10 mL[a]	
I	Citric acid, trisodium, dihydrate		Make solution A at 0.015 g/10 mL[a]. Make solution B by adding 1 mL of solution A + 9 mL water	
J	KH_2PO_4		0.014 g/10 mL[a]	
K	Taurine		0.013 g/10 mL[a]	
L Stability: 3 months, −20°C	Stock solutions of vitamins		Make 100 × stock in water containing 1 mL of 1 N NaOH/L	

[a]For these stocks, one can weigh out the approximate amount, divide by the concentration × 10 = volume of water to add, for example, if 0.35 g of glucose is weighed, then 0.35/0.5 × 10 = 7.0 mL water used

All stocks are stable for 3 months at 2–8°C except where noted. Frozen stocks should be thawed and used, and the remainder discarded

Table 16.2 Final preparation of media from stocks (Add the required amount of water to 0.050 g of BSA (for mouse embryos) or HSA for human embryos (see below) in a 14-mL Falcon tube)

Stock	Source (date) and/or lot #	Amount (mL)	✓ Added
H_2O		6.38	
A (salts)		1.0	
B ($NaHCO_3$)		1.0 or 0.16 for HEPES	
C (pyruvate)		0.1	
E (HEPES)		0.84	
F (amino acid stock solution)		0.1	
G (EDTA)		0.1	
H (glucose)		0.1	
I (citric acid—solution B)		0.01 (10 uL)	
J (KH_2PO_4) Use a higher concentration in stock if a higher final concentration is required, for example, 0.050 g/10 mL		0.01 (10 uL)	
K (taurine)		0.1	
L (vitamins)		0.1	
D (Ca lactate, pentahydrate) Always add this last and after thoroughly mixing all the other components. $CaCl_2.2H_2O$ and sodium lactate can also be used in place of calcium lactate		1.0	
BSA (or HSA, or another protein source; specify)		5 mg/mL, i.e., 50 mg for 10 mL	

1. Final total volume is 10 mL; adjust volume of water if different volume(s) of stock(s) is (are) being used to obtain different concentration of components
2. Mix thoroughly to dissolve all components
3. Filter sterilize
4. Add each of stocks to water, adding Ca salt stock last (otherwise, a precipitate will form). Mix thoroughly to dissolve all components. Add protein if not already done so as in step 1 above (e.g., HSA; 50 mg/10 mL = 5 mg/mL), and filter sterilize

Other specifications:
1. Osmolality: 265–280 mosmol/kg water
2. pH: 7.2–7.3, depending on concentration of $NaHCO_3$ and CO_2 used

Water: conductance 0.06 mΩ/cm; resistivity 18.0 MΩ/cm; silicate not detectable
Endotoxin: final product must be <0.1 EU/mL

Tips and Tricks for Using ART Culture Media

Some Dos and Don'ts for Using ART Culture Media

Dos

Measure the pH of each new lot of product received. If protein is added to the medium by the end user, do so and then equilibrate the completed medium under the atmosphere it will be used at for a sufficient time (minimum of 4 h but usually overnight) and measure pH at 37°C. The most practical way is to use the Pool protocol [45]. Using a suite of media from the same manufacture (e.g., fertilization, cleavage, and blastocyst media)—only measuring the pH in the fertilization medium and then assuming that if the pH is okay in fertilization medium, then it is okay in cleavage and blastocyst media—is not a good idea. This may not be so and is not a good practice. The medium has to have pH measured with protein included. The probe can be washed in a solution containing a protease to remove protein that may have stuck to the probe. An example of the protease-containing washing solution is Tergazyme [55], or buy the solution already made up [56]. When the pH of the media are known, it may then be necessary to adjust the CO_2 concentration in the incubator to obtain the desired pH in the fertilization and culture media.

Don'ts

Aliquot medium into 10-mL lots in 14-mL capped tubes and then store these in the refrigerator until the day of use. It is not good practice because handling the media like this makes them prone to microbial contamination, the pH will go up during this whole process, and when pH goes up, calcium carbonate can precipitate leading to problems as the $CaCO_3$ will not go back into solution when the pH is equilibrated down to the acceptable range.

Other practical suggestions for laboratory practice are given elsewhere [46, 48, 53].

Summary and Conclusions

To state the obvious, successful culture of human embryos, as with all other mammalian species, from the zygote stage through to D3 is an essential part of the ART process.

Unless this process is optimized, further development of the embryo is doomed. The main theme that I believe I have presented is that human embryos, especially during their first 3 days of development, can be cultured in a fairly diverse range of media and give reasonable take-home baby rates. Some aspects of media composition may be more important than others. Of equal, if not more, importance is the way the media are used. In this context, pH and temperature control and the concomitant decreased observation and disturbance of the embryo during in vitro development are extremely important practical aspects of culture that are often overlooked in ART laboratories. Prospective, proactive tracking of key laboratory performance indicators is an essential feature in successful ART programs [48]. To summarize, I use the statement "a good medium used poorly produces poor outcomes."

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Extended Culture in IVF

17

David K. Gardner and Michelle Lane

Abstract

With the advent of culture media based upon the nutrient composition of the human oviduct and uterus, and specifically designed to support the human embryo in vitro, it has become feasible to culture the pronucleate oocyte to the blastocyst stage as a routine procedure in human IVF. 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. The capacity to better select a viable embryo will continue to improve the safety of IVF to 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. The transfer of vitrified blastocysts in a nonstimulated cycle may ultimately be the way all future IVF is performed.

Keywords

Culture systems in IVF • Blastocyst culture • Sequential media in IVF • Embryo • Preimplantation • Viability

Introduction

The ultimate goal of human IVF is the birth of a healthy singleton conceived through the transfer of a single embryo. The benefits that single embryo transfer (SET) brings have been well documented [1], and SET has already been proven effective in selected groups of patients [2, 3]. The relatively recent development of more effective embryo culture systems has facilitated the establishment of higher pregnancy rates, by being able to maintain the inherent viability of the gametes and subsequent embryo in vitro, and has therefore facilitated the wider introduction of SET. Furthermore, with the advent of more physiological culture media, it is possible to grow the human embryo to the blastocyst stage as a matter of routine. Thus, it is now possible to consider embryo transfer at any stage of preimplantation development. In parallel with, innovations in embryo culture has been the development of many excellent scoring systems to quantify embryo morphology [4–6], which have greatly assisted in embryo selection for transfer. Concomitantly, we are currently witnessing the application of several omic platforms (i.e., genomics, transcriptomics, proteomics, and metabolomics) in human IVF. Such technologies are generating quantifiable metrics that will assist in improving all areas of IVF, including the culture systems themselves. However, it is potentially in the area of embryo selection that omics will make their greatest impact. Clearly, human IVF has taken a huge leap forward in recent years and is entering a new exciting period. The aim of this chapter is to outline advances in embryo culture, discuss the potential benefits of blastocyst transfer and how improvements in embryo culture have also impacted on embryo selection.

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Development of Culture Systems to Support the Human Embryo

Over the past two decades, there has been a resurgence of interest in the physiology of the preimplantation embryo and in the environment within the female reproductive tract. Subsequently, culture systems for the human embryo have become more physiological, typically based on the levels of carbohydrates within the female tract, and routinely contain amino acids. The increased efficiency of these new media is reflected in an increase in implantation and pregnancy rates that the IVF community has experienced over the past decade [7]. A key aspect of the advances in culture conditions has been the ability to culture human embryos to the blastocyst stage as a matter of routine.

Some of the inherent advantages of blastocyst culture and transfer are summarized in Table 17.1. For example, not all sperm or oocytes will give rise to a viable embryo [8]. By growing the human embryo beyond the cleavage stages, i.e., past embryonic genome activation [9], one is able to consider the embryo proper, as opposed to an 8-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 [10], 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 [11]. Although the human cleavage stage embryo can develop in the uterus, in vivo the cleavage stage embryo resides in the Fallopian tube until day 4 [12]. Consequently, asynchronous transfers are being performed in the majority of IVF cases. Furthermore, it has been documented in animal models that the environment within the female tract following gonadotropin treatment is not as supportive to embryonic development as a nonstimulated environment [13, 14]. 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 [15]. 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 transfer. Subsequently, the transfer of human embryos at the blastocyst stage has a sound physiological basis. Several of these advantages were perceived many years ago [16–18], 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.

What Systems Can Be Used to Culture the Human Embryo?

Early culture media used in human IVF tended to either be rather basic, containing just a few salts and carbohydrates, or were tissue culture media, which contained an excess of nutrients, 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 be Gardner and Lane [19]) such as those developed initially for the mouse and adapted for the human [20], were not effective in supporting human embryos past the 8-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 8-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 monolayers of oviduct or uterine epithelial cells [17] or specific cell lines [18]. Although the development of

Table 17.1 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 with 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 the 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
Ability to undertake trophectoderm biopsy rather than cleavage stage biopsy, thereby increasing the number of cells analyzed (typically 6–10) and overcoming ethical/religious concerns of sampling embryonic tissue
Excellent cryosurvival
Increase in overall efficiency of IVF

Table 17.2 Role of amino acids during early mammalian embryo development

Role	References
Biosynthetic precursors	[96]
Energy source	[97]
Regulators of energy metabolism	[36, 98]
Osmolytes	[99]
Buffers of pHi	[100]
Antioxidants	[101]
Chelators	[102]
Signaling	[103, 104]
Regulation of differentiation	[105, 106]

blastocysts was facilitated by such technology, co-culture was not widely adopted, nor its efficacy ever established in randomized controlled trials. Subsequently, co-culture was replaced with the current approach of the use of more physiological culture media [21].

A major breakthrough in the formulation of embryo culture media came from the determination that amino acids have a significant role to play during embryo development [22–26] (Table 17.2). In the presence of amino acids, embryo development in culture is temporally closer to that observed in vivo: more cells are allocated to the inner cell mass (ICM) of the resultant blastocysts, energy metabolism is better supported, there is reduced apoptosis, and, most importantly, embryo viability, as assessed by implantation and fetal development, is higher [26]. However, as we are 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, caused by working outside of the female reproductive tract. Amino acids are metabolized by embryos which subsequently release ammonium [25]. In vivo, one anticipates that this ammonium is removed by the epithelial cells of the female reproductive tract, and then passes through the circulation and 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 [25]. To add insult to injury, the amino acids themselves are labile at 37°C and spontaneously deaminate to release ammonium [25]. The significance of spontaneous ammonium build up is that it not only retards mouse and human blastocyst development in culture [27], but it has been associated with subsequent fetal retardation and neural tube defects in mice [26]. Furthermore, there appears to be a link between the concentration of ammonium in serum and the induction of fetal oversize in sheep [28, 29]. Therefore, it is advisable to renew the culture medium used at least every 48 h in order to circumvent the toxicity of ammonium. 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 Gardner and colleagues measured the ammonium production by human blastocysts [30], it was found that the embryo produced significant amounts of ammonium, presumably through amino acid metabolism and transamination. The significance of these findings is that whatever culture system is employed, it is imperative to renew the medium after 48 h to ensure minimal accumulation of embryotoxic ammonium. From a practical point of view, therefore, the amount of work, cost, and embryo manipulations required is the same whether one is working with sequential media or a monophasic system (i.e., one medium formulation for the entire preimplantation period).

Of significance, the female tract provides gradients of nutrients, both carbohydrates [31] and amino acids [32, 33], which are involved in the regulation and production of energy by the developing embryo. The significance of these nutrient gradients to the embryo in culture warrants further research as existing data on the mouse indicates that such gradients in vitro impact embryo viability following transfer. For example, when the mouse zygote is cultured to the 8-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) [34]. These data support the hypothesis that the physiology of the developing conceptus is temporally regulated by concentration gradients of nutrients available [35].

One Medium or Two?

Sequential media were developed based on these changing environments within the female reproductive tract and to accommodate the changes in nutrient requirements and metabolism of the developing embryo [36, 37]. However, 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 [38]. We, therefore, decided to perform an analysis of the efficacy of such media types on the development of mouse pronucleate oocytes in vitro, and to compare their rates of development to in vivo developed embryos [39]. The media chosen were Whitten's (one of the very first mouse embryo culture media)

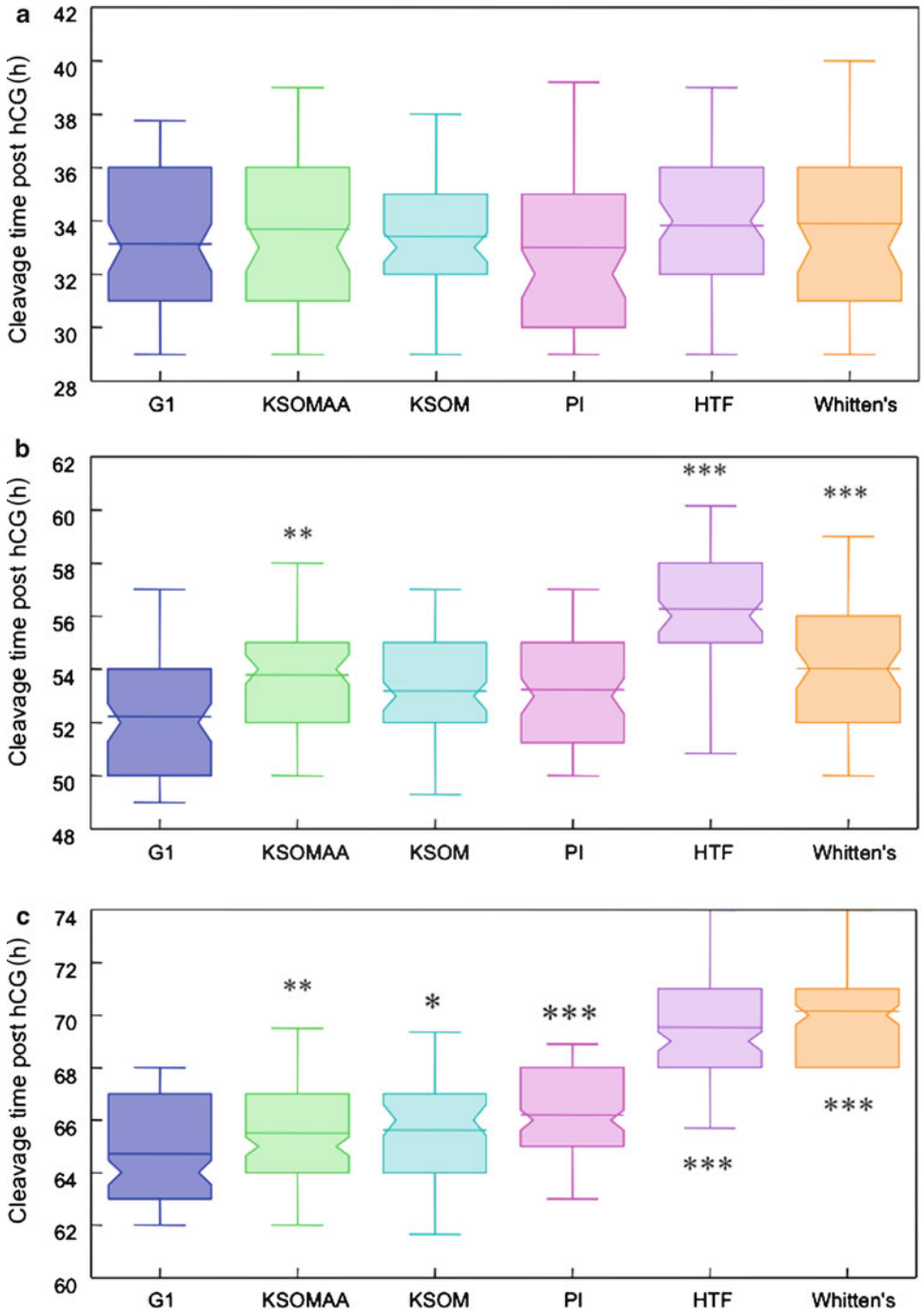

Fig. 17.1 Effect of culture media on cleavage times in hours from the zygote to the 8-cell stage. (**a**) Cleavage from the 1- to 2-cell stage. (**b**) Cleavage from the 2- to 4-cell stage. (**c**) Cleavage from the 4- to 8-cell stage. Notches represent the interquartile range (50% of the data); whiskers 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; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

[40], HTF (one of the first simple media developed for human embryos) [20], P1/Blastocyst (a glucose- and phosphate-free modification of HTF for 48 h after which the embryos are transferred to Ham's F10) [41], KSOM (a computer-optimized monophasic medium lacking amino acids) [42], KSOMAA (a computer-optimized monophasic medium with amino acids) [43], and G1G2 (a sequential media system based around the composition of the human female reproductive tract) [44]. All media were made with the same chemicals and water, and all embryos were transferred to fresh media after 48 h. All cultures were performed at 5% oxygen. There was no difference in cleavage time from 1- to 2-cell between any media (Fig. 17.1a). From 2- to 4-cell, embryos cultured in KSOMAA, HTF, and Whitten's media were significantly slower than those in medium G1 (Fig. 17.1b). From 4- to 8-cell, embryos cultured in all media were significantly slower than in medium G1 (Fig. 17.1c). Rates of compaction were significantly greater in embryos cultured in G1 (74.4%) compared to all

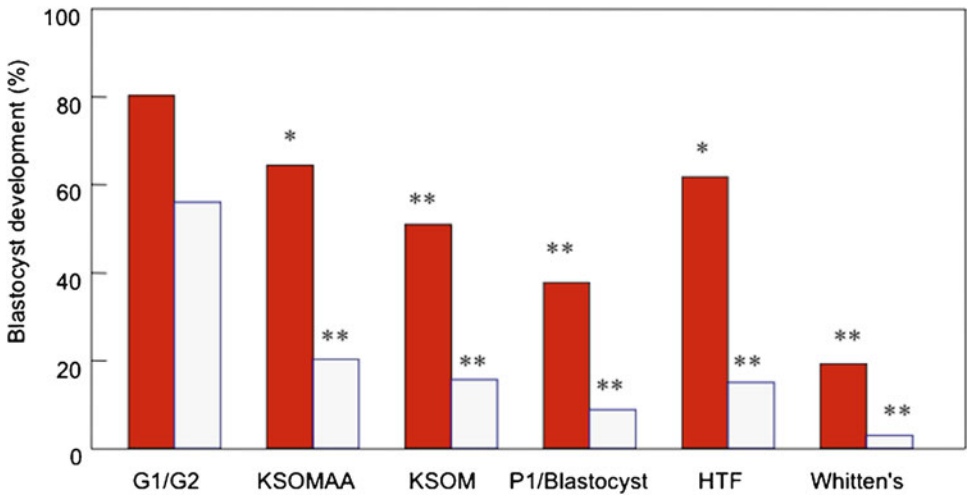

Fig. 17.2 Effect of culture conditions on blastocyst development at 94 h post-hCG. *Red bars* represent total blastocysts. *White bars* represent expanded blastocysts. Significantly different from G1/G2 embryos, $*P<0.05$, $**P<0.01$. At 94 h post-hCG, 82% of in vivo developed embryos were at the blastocyst stage

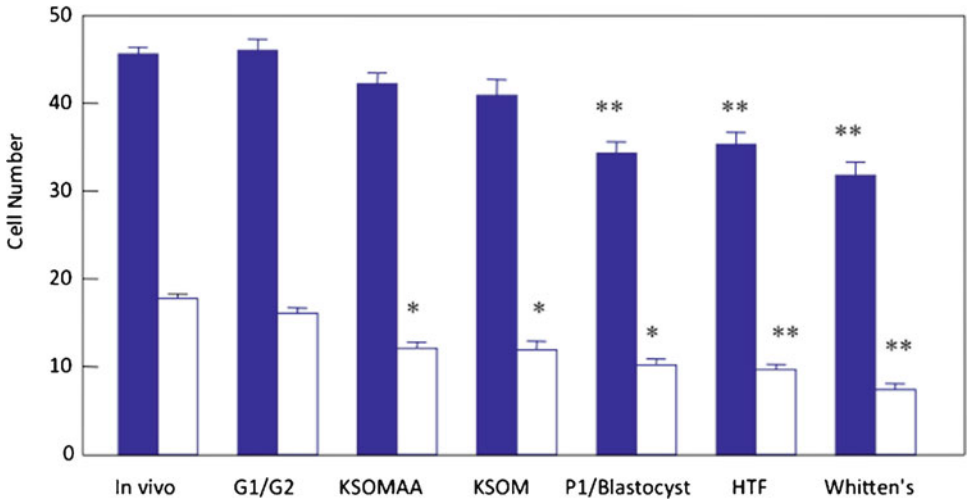

Fig. 17.3 Effect of culture conditions on blastocyst cell number and ICM development. *Blue bars* represent total cell number. *White bars* represent inner cell mass cell number. Significantly different from in vivo, $*P<0.05$, $**P<0.01$

other media: KSOMAA (60.2%), KSOM (65.9%), P1 (66.0%), HTF (26.4%), Whitten's (0.0%). Rates of blastocyst development were similar to in vivo developed embryos in media G1/G2 (Fig. 17.2). Blastocyst development in all other media was significantly slower to that obtained in vivo or in media G1/G2. Total blastocyst cell number was similar to in vivo blastocysts in media G1/G2, KSOMAA, and KSOM (Fig. 17.3). However, only the ICM development in G1/G2 blastocysts was equivalent to in vivo-developed blastocysts (Fig. 17.3). The data obtained demonstrate that embryos cultured in sequential media develop quicker than those in a monophasic system and that 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 [45].

Sequential media have now been proven to be highly effective in clinical settings. Importantly, assessment of the literature using an evidence-based Cochrane review [46] has determined that the benefit of blastocyst culture over cleavage stage culture is only evident in a sequential culture system, thereby indicating that there is a compromise in developmental competence when embryos are grown beyond the cleavage stage in a single medium. This finding for

human IVF cycles mirrors the literature on animal embryos that have established developmental outcomes more similar to in vivo results after culture in sequential style culture media compared to a monophasic culture system [45].

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 [45]). Furthermore, it is imperative that sufficient resources be made available for quality control [47] and quality management [48], without which the lab 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 lead 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% [49, 50], whereas the oxygen concentration in the oviduct of hamster, and rhesus monkey is ~8% [51]. 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 [51].

Significantly, it has been demonstrated that optimum embryo development of several mammalian species occurs at an oxygen concentration at or below 10% [52–54]. Furthermore, it has been documented that mouse embryos cultured to the blastocyst stage in the presence of 20% oxygen have altered gene expression and a perturbed proteome compared to embryos developed in vivo [55, 56]. In contrast, culture in 5% oxygen had significantly less effect on both embryonic gene expression and proteome. Recent clinical data supports the move to the routine culture of human embryos in a reduced oxygen environment [57, 58]. Furthermore, data obtained using time-lapse incubation strongly supports the notion that at no time during the preimplantation period should the embryos be exposed to 20% oxygen [59].

Considering the physiology of the reproductive tract and the beneficial effects of using a reduced oxygen concentration as determined in controlled studies, it is advisable to culture embryos at low oxygen concentrations. This can easily be achieved with the modern tri-gas incubators or by using a premixed cylinder to purge a modular chamber/direct contact incubation system.

Implementation of Blastocyst Culture

Blastocyst transfer has now been used successfully to treat patients with poor-quality embryos [60, 61], patients with multiple IVF failures [62–64], patients with low numbers of oocytes and embryos [65], and oocyte donors [66]. 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 [67–69].

Parental factors also affect blastocyst formation and pregnancy-implantation rates. As maternal age increases, both the number and quality of the oocytes retrieved is reduced, thus affecting the outcome of blastocyst transfer [70, 71]. The success of blastocyst culture has also been shown to be under paternal influence [72]. 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 [73].

Gardner and Balaban [74] reviewed 16 prospective randomized trials using sequential media examining the effect of day of transfer on IVF cycle outcome [63, 68, 69, 75–87]. Of these 16 trials, seven reported a significant benefit in outcome as defined by either increased implantation rate or pregnancy rates (one 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 [79]. The remaining eight trials reported no difference in implantation rate with respect to day of transfer.

The move to blastocyst culture has had a significant impact on reducing the number of embryos required for transfer, thereby greatly reducing the incidence of high-order multiple gestations. Although triplets can be confined to the archives of IVF, the overall multiple pregnancy 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 (SBT). In the first prospective randomized trial of single vs. two blastocyst transfers, in a population of patients with a day 3 FSH ≤10 mIU/mL and at least ten follicles >12 mm in diameter on 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 [3]. Significantly, when two blastocysts were transferred, the pregnancy rate rose to 76%, but with a 47.4% incidence of twins.

Therefore, the move to 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 were 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 [87–89].

Blastocyst Selection for Transfer

As the embryo develops and differentiates into the blastocyst, with its distinct cell types (the ICM and trophectoderm), it becomes feasible to give a more detailed grading to the human embryo. An alphanumeric system [90] has been widely adopted and has proven valuable in assessing blastocyst viability prior to transfer [6, 91]. Figure 17.4 shows a human blastocyst that has formed a well-defined ICM surrounded by a cohesive multicellular epithelium, the trophectoderm. Such an embryo from patients <38, 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 analysis, it is evident that even blastocysts with the same morphology have different physiologies [30, 92]. 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 SET. Similarly, the advent of highly sensitive molecular techniques is making karyotyping the blastocyst a reality in an IVF cycle [93]. Such molecular analyses require the cryopreservation of the embryo. The capacity to perform these analyses is supported by developments in blastocyst vitrification which have facilitated safe and effective blastocyst cryopreservation [94, 95].

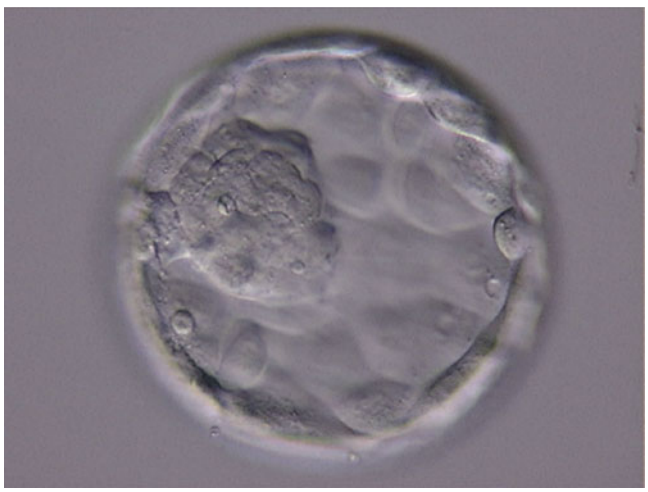

Fig. 17.4 Photomicrograph of a human blastocyst on day 5 of development. Such a blastocyst is graded as 4AA

Conclusions

With the advent of more physiological culture media, together with better laboratory conditions and management, it is 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. The capacity to better select a viable embryo will continue to improve the safety of IVF to 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. The transfer of vitrified blastocysts in a nonstimulated cycle may ultimately be the way all future IVF is performed.

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In Vitro Maturation of Human Oocytes

18

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Abstract

Although the initial live birth resulting from human in vitro fertilization (IVF) was achieved by natural-cycle IVF, this procedure was gradually replaced by ovarian stimulation IVF because it was found that the number of oocytes retrieved related to the embryos available for transfer which, in turn, directly affected successful pregnancy. However, repeated ovarian stimulation by gonadotropins has certain drawbacks including a higher risk of ovarian hyperstimulation syndrome and the concerns about a possible increased risk of ovarian, endometrial, and breast cancers. Therefore, there is a growing interest in natural-cycle IVF and in vitro maturation (IVM) treatment. Recovery of immature oocytes followed by IVM is a potentially useful treatment for women with infertility. The method is particularly effective for infertile women who have numerous antral follicles due to polycystic ovaries or polycystic ovarian syndrome. Today, given the efficiency of IVF and improvements in the culture system, natural-cycle IVF is more suitable for women undergoing infertility treatment. One very attractive possibility for enhancing the successful outcome of natural-cycle IVF treatment is combining it with immature egg retrieval and IVM. The use of IVM technology can thus be broadened to treat women suffering from all causes of infertility. The aim of this chapter is to share our experience and protocols with the assisted reproductive technology fraternity.

Keywords

Immature oocytes • IVM • Fertilization • Pregnancy • Natural-cycle IVF/M

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 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

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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 was 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].

Indications for IVM Treatment

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.

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 1,554 oocytes collected were in vivo matured.

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.

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%.

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].

Step-by-Step Protocol for IVM Treatment

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 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.

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 bloodstained 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.

Laboratory Procedure

Similar to IVF, the follicular aspirate is first examined under a stereomicroscope to identify cumulus-oocyte complexes (COC). Since identification of small immature oocytes surrounded by scarce granulosa cells which are not widely

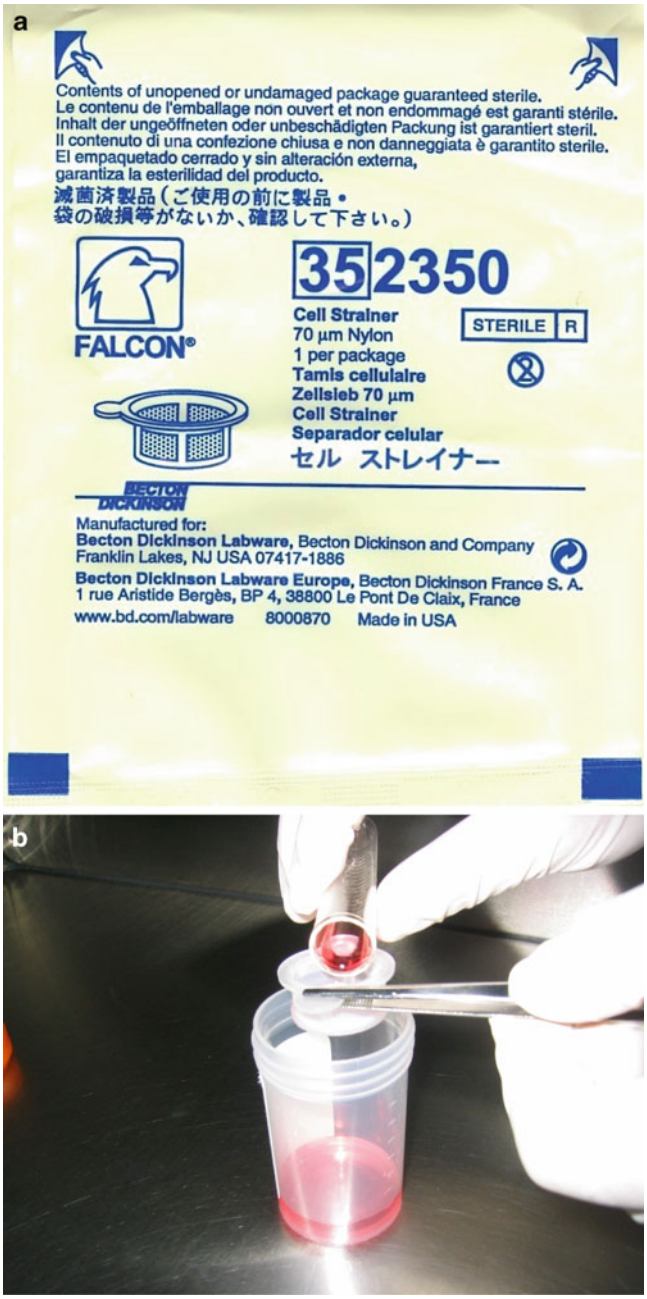

Fig. 18.1 (**a**) Labeling on Cell Strainer (Falcon, Becton Dickinson and Company, USA); (**b**) follicular aspirates are filtered with a Cell Strainer

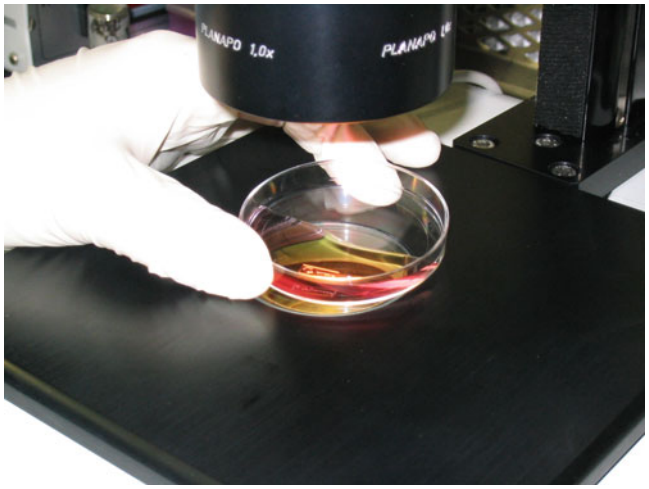

Fig. 18.2 Sliding technique for observation of oocyte maturity. COCs are allowed to slide slowly from one side to the other on the bottom of the Petri dish, while being observed under the stereomicroscope

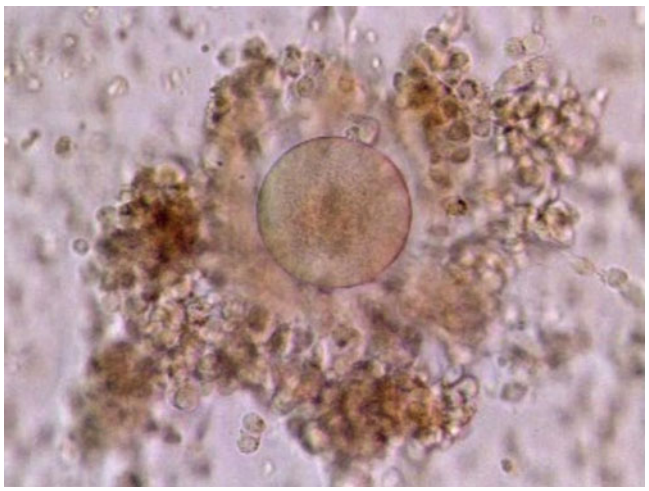

Fig. 18.3 During COC sliding, it is possible to observe clearly whether or not the oocyte has extruded a first polar body (1PB) into perivitelline space (PVS)

dispersed is more difficult with the conventional technique as they can be overlooked, follicular aspirate is filtered through a nylon mesh strainer (Fig. 18.1) with 70 µm pores after 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 oocyte. 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. 18.2). 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. 18.3). 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. 18.4) containing 1 mL oocyte maturation medium supplemented with a final concentration of 75 mIU/mL FSH and

Fig. 18.4 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

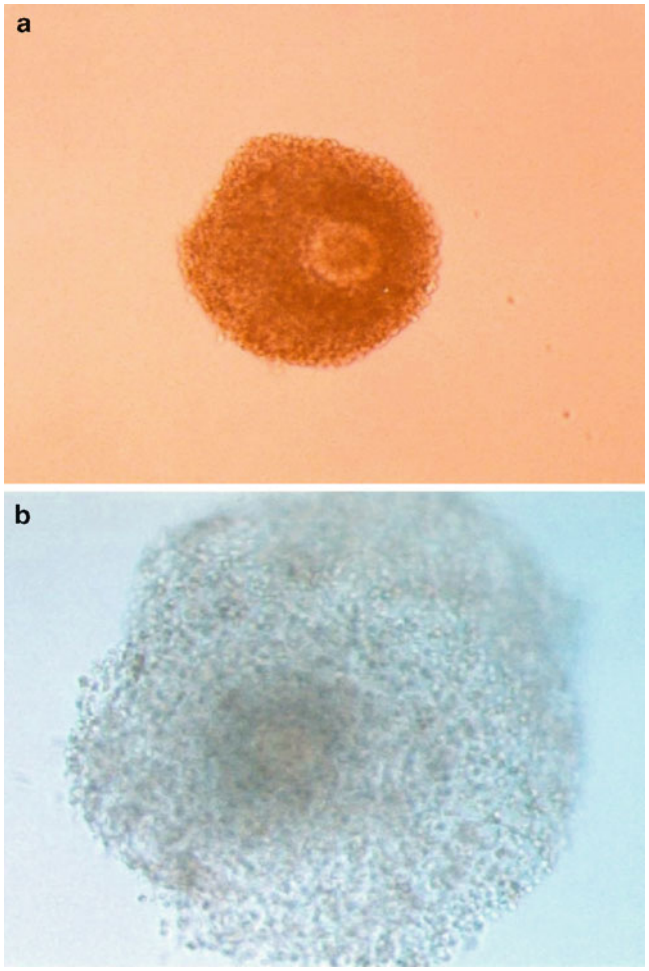

Fig. 18.5 Morphological changes of immature COC before and after culture in the oocyte maturation medium. (**a**) Immature COC immediately after retrieval from the ovary; (**b**) the same COC after 24 h of culture in the oocyte maturation medium. Note that the cumulus mass has almost doubled in size compared with its mass before culture (two photos with the same magnification)

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 for equilibration at least 2 h before immature oocyte retrieval (particularly, it can be made 1 day before). Some commercial available oocyte maturation medium does not contain 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. 18.5), 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 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 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.

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 above-mentioned concerns about zonal hardening [50].

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.

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].

Pregnancy Loss

Biochemical pregnancy loss rates were similar among 1,581 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 ($p<0.01$) [56]. 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 [57–59]. Arguably, the higher incidence of PCOS in IVM patients in this series can be the reason of the higher miscarriage rate 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.

Obstetric Outcome

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 Jan 1, 1998, to Dec 31, 2003 [54]. 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.

Congenital Abnormalities and Physical and Neuromotor Development

In the above-mentioned 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 [54]. 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.

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 [60]. Another study of 46 IVM babies born to 40 women reported similar findings [61]. 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 [60, 61]. Currently available data seem reassuring and do not suggest an increased risk of congenital malformations and physical or neurological developmental delay in IVM children.

Summary

IVM is a relatively 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. 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 benefit from advantages of IVM.

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 by our team [62, 63]. IVM has enabled successful treatment of patients with empty follicle syndrome in previous stimulated IVF cycles [64]. Patients can undergo several IVM cycles, and we previously reported a series of patients who achieved repeated live births with IVM treatment [65].

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In Vivo Embryo Culture Device

19

Claude Ranoux

Abstract

The INVO procedure using the INVOcell device is a simple technique of human assisted reproduction. The fertilization of the oocytes and the early development of the embryos occur in the INVOcell device placed into the vaginal cavity for an in vivo incubation. The vaginal cavity plays the role of the CO_2 incubator by supplying the temperature and gas environment needed for the embryo development. Capital equipments found in a complex IVF laboratory are not necessary as gametes and embryos are not stored in the laboratory. The INVO procedure can be performed in a medical office setting. INVO is a proven technology that showed clinical pregnancy rates equivalent to conventional IVF when using a prototype. This chapter presents the preliminary results obtained in combining mild ovarian stimulation with INVO using the new designed INVOcell device. The low cost of the INVO procedure and its large availability allow the treatment of a significant portion of infertile couples in the world who could not access any reproductive technology before. The more natural in vivo conception of the embryos with the participation of the patients has generated more interest in INVO from the infertile population and specialized physicians.

Keywords

INVO procedure • INVOcell device • Low cost • Vaginal incubation • Office setting procedure

The intravaginal culture (IVC) called the INVO procedure is a unique option for patients seeking treatment in infertility. The INVO procedure was created to simplify the process of in vitro fertilization (IVF) and early embryo development and reduce the sophisticated laboratory instrumentation required. Mild ovarian stimulation or natural cycle combined with the INVO procedure makes possible the treatment to infertile couples in a medical office setting such as the office of an infertile specialist [1], a satellite unit of a reproductive center, or in some areas an ob-gyn's office. The INVO procedure using the INVOcell device substitutes the CO_2 incubator used in conventional IVF with the vaginal cavity of the patient.

History of the Intravaginal Culture Also Called INVO

Since the birth of Louise Brown, the first baby born by IVF in 1978, several scientific milestones have been accomplished in assisted reproductive technologies (ART). Most of these scientific advances such as controlled ovarian hyperstimulation, embryo cryopreservation, and intracytoplasmic sperm injection (ICSI) have not only increased the embryo implantation rates but also created clinical, ethical, and legal issues and dramatically complicated the IVF procedure.

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Table 19.1 Initial results of INVO using the prototype device

Number of publications	Countries	Number of INVO cycles	Clinical pregnancy rate/cycle (%)
9	Austria, France, Germany, Japan, Netherland, UK, USA	815	19.6

These complicated technical advances have contributed to increased costs of IVF. The high cost of IVF has resulted in rejection of reimbursement by the insurance companies thus restricting IVF access to infertile couples who can afford to pay out of pocket. These technical advances have also contributed to the creation of governmental regulations for ART in many countries. All these factors have slowed the expansion of IVF technologies—a major factor that explains why, after more than 30 years of existence, so many infertile couples cannot and do not receive IVF treatment. The INVO procedure performed by a trained physician and embryologist in an office or satellite unit becomes accessible to many more insured and noninsured infertile couples.

Principle

The INVO procedure consists of utilizing the vaginal cavity environment for the oocyte fertilization and early embryo development [2]. The INVOcell device is specially designed for the INVO procedure [3]. The vaginal cavity provides the pCO_2, pO_2, and temperature for the culture of the gametes and the embryos [4]. The INVOcell device is permeable to gas and allows the equilibrium between the pCO_2 of the vagina and the pCO_2 of the culture medium. This system maintains the pH of the culture medium between 7.2 and 7.4 during the entire period of vaginal incubation. This in vivo fertilization and early embryo development involves the participation of the patient giving a more natural approach to the assisted conception.

Discovery of the Intravaginal Culture

The INVO technique was discovered around 1985. The laboratory IVF incubators at the time had CO_2 distribution controlled by a bead system. The rate of CO_2 mixed with the air was very imprecise and had peaks of low and high CO_2 concentrations that resulted in major variations in the pH of the culture medium. When cells or mouse gametes were placed in the same incubator in plastic tubes filled with culture medium and hermetically closed, the passage of CO_2 through the wall of the sealed plastic tubes altered the peaks of CO_2 and dramatically reduced the variations of pH in the culture medium. To simplify the process, the natural vaginal cavity providing CO_2 and O_2 was used. There was originally a concern of potential lesions of the uterine cervix from the prototype device during incubation that could interfere with the quality of the embryo transfer. This was eliminated by tests that demonstrated no lesions or interference. This was later confirmed by the results of the first INVO procedures showing comparable embryo implantations to conventional IVF.

Prototype and Initial Results with the Prototype

Prototype

During the initial cases and publications utilizing the INVO procedure, a prototype which was a simple plastic tube was used [2]. The tube chosen showed the best sealed closure among several prototypes that were initially tested. The prototype device was filled with culture medium first to avoid air bubbles. Air bubbles could be caught by the viscous cumulus of the mature oocytes causing them to float up and decreasing the chances of fertilization. These air bubbles could modify the pH of the culture medium. With the prototype tube, oocytes could also be lost in the overflow during the closing of the prototype.

Initial Results with the Prototype

Several publications in international medical journals [1, 2, 4–10] were issued at this time reporting the results obtained by INVO using this prototype device (Table 19.1).

Some of these publications clearly indicated equivalent pregnancy rates between INVO using the prototype device and conventional IVF [2, 5, 9].

From the Prototype to the Improved INVOcell Device

The first practitioners using the INVO prototype device had many difficulties. The device could only be opened and closed once. There were observations of variations in pH of the culture medium, bacterial contamination, accidental openings of the prototype in the vaginal cavity, loss of embryos, and increased risk of vaginal bacteriosis. A new device, the INVOcell made of three parts, was designed to address all these identified technical problems [3].

Fig. 19.1 Inner chamber and open outer rigid shell of the INVOcell device

Fig. 19.2 Fully assembled INVOcell placed in the retention system and ready to be positioned in the vagina

The Inner Chamber

The inner chamber houses the culture medium and the gametes. A rotating valve allows several openings and closings of the inner chamber without introduction of air or contamination of the culture medium. The rotating valve has a small orifice at a bottom of a small well which prevents major variations in pH of the culture medium and loss of gametes due to possible overflow while filling. The volume of the chamber has been reduced to 1.08 mL from the initial 3 mL. At the bottom of the main chamber, a microchamber collects the embryos after incubation. This microchamber allows direct observation and selection of the embryos without transfer to a culture dish. Loading of the embryos can be done directly from the microchamber with the embryo transfer catheter.

The Outer Rigid Shell

The outer rigid shell protects the inner chamber from vaginal contaminations and keeps the inner chamber sterile (Fig. 19.1). It has a smooth external surface to prevent any lesions or irritations of the vagina and cervix during the 3 days of vaginal incubation. The wall is permeable to CO_2 and O_2. The rigid shell could be grasped if necessary with a forceps to remove it from the vagina. A locking position prevents any unexpected device opening during vaginal incubation.

The Retention System

The retention system has also been improved. Holes have been perforated in the membrane of the diaphragm for the elimination of the vaginal secretions during incubation (Fig. 19.2). Tests of comfort and retention, requested by regulatory agencies for approval of the device, were performed using the INVOcell and its retention system. The INVOcell device does not cause any discomfort or irritation of the vaginal cavity and does not increase incidence of bacterial vaginosis from the 3 days of incubation. No device expulsion was observed when the retention system was used. These results were confirmed by the first INVO procedures using the INVOcell.

The INVO Cycle

INVO Cycle

The indications for using an INVO cycle are similar to the indications using conventional IVF. INVO is not recommended in severe oligoasthenoteratozoospermia. All other indications can be treated by INVO.

Natural Cycle or Mild Ovarian Stimulation Protocols

Current stimulation protocols use the association of gonadotropin-releasing hormone (GnRH) agonist and high doses of human menopausal gonadotropin (hGM) or follicle-stimulating hormone (FSH). These protocols recruit a lot of follicles and show complications such as severe ovarian hyperstimulation syndromes (OHSS) as well as multiple pregnancies with premature deliveries, birth defects, and maternal complications. The use of these protocols and their complications represent a very costly burden for society. The governments of several countries and specialized associations such as the American Society for Reproductive Medicine (ASRM) have developed regulations and guidance concerning the numbers of embryos to transfer. This has contributed to a returned interest in natural cycle and mild stimulation protocols that produce less embryos and are safer

for the female. The introduction of GnRH antagonists [11, 12] or indomethacin [13, 14] in preventing premature LH surges and ovulations has also contributed to the reintroduction of the natural cycle and mild ovarian stimulation protocols. Mild stimulations and natural cycle protocols with the INVO procedure contribute equally to the simplicity, low complication rates, and low cost of the INVO cycle.

Modified Natural Cycle

The monitoring of natural cycle is simple and inexpensive. Generally, an average of four rapid immunoenzymatic blood assays, when available, and two or three ultrasound exams starting at day 8 precede the retrieval. GnRH antagonist (0.25 mg daily) or indomethacin (50 mg 3× per day, as used in Dr. Lucina's study and discussed later) is started at day 8 or when the leading follicle reached 15 mm and is used to prevent premature ovulation. Triggering of ovulation is performed by the injection of 5,000 IU of human chorionic gonadotropin (hCG) when the size of the follicle reaches 18 mm and the estradiol 180 pg (pictogram)/mL, when dosage is available.

Clomiphene Citrate Protocol

The monitoring of the stimulation and the control of the premature ovulation are identical to the modified natural cycle. Induction of the ovulation is based on the same follicular size (18 mm) of the dominant follicle and is done using 10,000 IU of hCG. Clomiphene citrate (CC) is generally used at a dose of 100 mg/day from day 3 to day 7. In developing countries, an aromatase inhibitor, letrozole (2.5–7.5 mg/day), is used from day 3 to day 7 and is preferred to CC due to its lower antiestrogenic action and better embryo implantation, and it requires less exogenous gonadotropins [15] (letrozole is not allowed in the USA and Europe). Gonadotropin, hMG, or FSH, 75 units, may be added every day starting on day 3 or day 5 of the cycle depending of the number of follicles to recruit. Generally, two to seven oocytes are retrieved.

Luteal Phase Support

Luteal phase is usually supported by progesterone (200–600 mg a day) started after the follicle retrieval and continued until the 10th week of pregnancy when the placenta takes over the progesterone secretion. Estradiol support (4 mg a day) is also used in association with progesterone.

Follicle Retrieval

Transvaginal follicle aspiration using ultrasound vaginal probe is performed 36 h after hCG injection to get the best oocyte maturity from the dominant follicle(s). Mild ovarian stimulation protocols recruit few follicles, allowing a short retrieval time. The use of conscious sedation makes the retrieval procedure well tolerated by the patient without the need for general anesthesia [1, 2]. A pump with control of vacuum pressure (120 mm hg) is recommended for the follicle aspiration; if not available, a follicular aspiration using 10-mL syringes can be done [2].

INVO Procedure

In INVO, the vagina provides the proper incubation temperature and the correct CO_2 supplementation required for embryo development. The INVOcell device has been designed to maximize the transfer of CO_2 present in the vaginal cavity to the culture medium, maintain the pH of the culture medium during the period of incubation, and reduce the quantity of O_2 transfer to the medium. The INVOcell eliminates the need for a complex laboratory and simplifies all the steps of the assisted fertilization and early embryo development.

Sperm Preparation

In the INVO procedure, the sperm preparation takes place before oocyte retrieval, so the oocytes can be inseminated immediately after the retrieval procedure without major exposure to a detrimental environment.

Sperm collection: Collection is generally performed by masturbation; if the collection is done by intercourse, a non-toxic condom should be provided.

Sperm washing and selection: Currently, the "swim-up" and the density gradient separation are the techniques used for sperm preparation. These two different techniques have the same principles wash the sperm to eliminate the seminal fluid and components which may interfere on fertilization and select the most motile spermatozoa. Density gradients have demonstrated a better selection of motile spermatozoa in oligoasthenozoospermia.

Insemination Using the INVOcell

Insemination using the INVOcell is performed immediately after oocyte retrieval. This point is very important, especially when the facility performing the INVO procedure does not have a CO_2 incubator or any CO_2 supplementation. It minimizes the exposure of the oocytes to the ambient atmosphere that is low in CO_2 and rich in oxygen (O_2). To maintain the proper pH of the culture medium during the short exposures to the ambient atmosphere, it is recommended to use HEPES media.

INVOcell preparation: The INVOcell parts, the inner chamber, the rigid outer shell, and the retention system are prewarmed before use. The inner chamber is rinsed with culture medium and then filled with 1.08 mL of fresh culture medium. The inner chamber is closed and replaced in

the incubator until the placement of the gametes. Several culture media have been used successfully with the INVOcell; they have to be bicarbonate-buffered. The media have to support 3 days of culture. Media with the addition of small amounts of phenol red are recommended especially when no CO_2 incubator is available as the phenol red is a very sensitive pH indicator.

Placement of the gametes: The fraction of motile spermatozoa is introduced first into the inner chamber. A total number of *30,000 motile spermatozoa* are used for insemination regardless of the number of eggs placed in the device. In case of oligoasthenoteratozoospermia, the sperm number may be increased to 50,000 motile spermatozoa. The oocytes, immediately after retrieval, are placed in warmed culture medium with HEPES. When all the oocytes have been collected, they are rinsed in one drop of buffered bicarbonate culture medium to eliminate the HEPES and then are transferred in the inner chamber. The rotating valve is then closed. The inner chamber is placed into the bottom of the outer rigid shell. The outer rigid shell top is then closed in a locked position. The device is now ready for placement in the vagina (Fig. 19.2). If for any reason the patient is not ready for the placement of the device, put the device back into the incubator until it can be placed in the vaginal cavity. It is essential to transfer the device into the vaginal cavity as soon as possible after insemination; the vaginal cavity provides the correct gas environment.

In Vivo Embryo Culture

The fully assembled INVOcell device should be inserted into the vagina manually by the physician. The use of a speculum makes the process more difficult for the physician and very uncomfortable for the patient.

Vaginal incubation: The device is designed to be held in the fornix or in front of the cervix during the 2 or 3 days of incubation and maintained in place using the retention system. It is recommended that the couple have no intercourse during the period of incubation. Female patients can shower, but no bath, swimming, or vaginal douche is allowed due to the potential changes in vaginal temperature that could affect incubation. Normal daily activities can be performed during the 3 days of incubation. Instructions including recommendations are provided to the patient.

Embryo(s) Transfer

The embryo transfer is generally performed 3 days after the insemination at the reproductive unit.

Device removal: The device and retention system are removed manually by grasping the ring of the retention system and pulling them out. The device is rinsed with prewarmed saline solution to clean off the vaginal secretions.

Fig. 19.3 Fully assembled INVOcell in vertical position and inner chamber positioned in the holding block for observation

Embryo Settling

The laboratory has a CO_2 incubator: The outer rigid shell is opened and discarded. The inner chamber is placed in a vertical position in the holding block in the CO_2 incubator for 15 min. During this time, the embryo(s) settle at the bottom into the microchamber.

The laboratory has no CO_2 incubator: If no CO_2 gas is available, keep the inner chamber in the outer rigid shell. The layer of gas captured between the inner chamber and the outer rigid shell will help to maintain the pH and temperature of the medium during embryo sedimentation. Place the cleaned device in a plastic sterile container in a vertical position in the incubator for 15 min. Just before the embryo observation, discard the rigid shell and place the inner chamber in the holding block (Fig. 19.3).

Embryo(s) observation and selection: The holding block containing the inner chamber is removed from the incubator and put on the microscope stage. The holding block has been designed not only to maintain the correct temperature and pH of the culture medium in the inner chamber but also to allow microscopic observation of the gametes and embryos directly through the wall of the inner chamber. In the holding block, the inner chamber is immersed in mineral oil which will eliminate irregularities of the device allowing clear viewing of the embryos directly from the microchamber. With the block in the vertical position, the oil is located internally in a reservoir (6.5 mL). During the microscopic observation, the block is flipped in a horizontal position. The microchamber is centered in the observation window and covered by the mineral oil coming from the reservoir of the block. When the embryo(s) have been located, the magnification is increased to grade and evaluate the stage of their development (two to eight cells). It is recommended that no more than two quality embryos be

Table 19.2 Results of the prelaunch clinical trial using the INVOcell

Groups	Cleavage rate (%)	Clinical pregnancy	
		Rate per cycle	Rate per transfer
Group 1 ≤10 Oocytes retrieved	52.20	31.80% (7/22)	38.90% (7/18)
Group 2 >10 Oocytes retrieved	49.30	11.70% (7/60)	13.50% (7/52)
Total	49.90	17.10% (14/82)	17.10% (14/82)

transferred to minimize the risk of multiple pregnancies. However, in some special circumstances, this number may be increased to three after discussing with the couple and after obtaining their agreement.

Embryo Transfer Catheter Loading

Embryo(s) can be loaded directly from the inner chamber: The embryo transfer catheter filled with culture medium is placed through the orifice of the open valve of the inner chamber positioned in the holding block. The embryo(s) can be visually selected and withdrawn from the microchamber into the transfer catheter by moving the syringe plunger attached to the transfer catheter up and down.

Embryo(s) loaded from a culture dish: A volume of 100 μL is aspirated from the microchamber under microscopic observation using a long pipette tip. This volume is transferred into a culture dish containing HEPES culture medium for observation and selection of the embryos for transfer. The selected embryos are then rinsed in fresh medium and loaded as classically into the embryo transfer catheter.

Embryo transfer into the uterus: The embryo transfer is performed using ultrasound guidance and an abdominal transducer to visualize the correct position of the catheter in the uterus. Any bleeding should be carefully avoided during the embryo transfer as it negatively impacts the prognosis of the procedure.

Preliminary Results

Prelaunch Trial

During the development of the new INVOcell device and its clearance by regulatory agencies, a lot of tests have been performed including a clinical trial. Results of this trial are shown in Table 19.2. A high number of oocytes, over ten per retrieval, were obtained in almost three-fourths of the cases (group 2) as all the stimulation protocols used GnRH agonist with high doses of gonadotropins. By agency request, no more than ten eggs could be placed in the INVOcell. Therefore, the embryologist had to select ten oocytes with the best maturity among the 20–30 retrieved oocytes. This factor certainly explains the low pregnancy rate obtained in group 2. In group 1, patients received the same regimen of drugs but developed only ten or less than ten oocytes. In this group of low responders with the highest pregnancy rate, all the oocytes were placed in INVOcell without preselection by the embryologist.

Only the two best embryos were transferred, resulting in 14 clinical pregnancies with 12 births of normal babies and only one set of twin.

Table 19.3 Results of the first postlaunch INVO procedures using INVOcell

Countries	Number of cycles	Clinical pregnancies (rate)
Austria, Bolivia, Brazil, Ecuador, India, Mexico, Nicarragua, Pakistan, Panama, Peru, Spain, Turkey, Venezuela	457	128 (28%)

Table 19.4 Results of the clinical trial performed at CECOLFES

Number of cycles	125
Number of embryos per transfer	2.4
Number of single pregnancies	40
Number of multiple pregnancies	10
Total number of pregnancies	50
Clinical pregnancy rate/cycle	40%
Clinical pregnancy rate/transfer	43.9%[a]

[a] Eleven embryo transfers were not performed, nine due to poor or no fertilization. In the two last cases, the husbands were not available at the embryo transfer, and embryos were cryopreserved by vitrification

Postlaunch INVO Cycles

Since the launch of the product at the end of 2008, several hundred procedures have been done with the INVO procedure using the INVOcell. Only results of one fraction of these procedures are reported in Table 19.3, the ones for which data have been obtained and confirmed. These procedures were performed in different countries in reproductive centers as well as in newly created INVO units.

Clinical Trial at CECOLFES (Colombia)

In addition to these INVO procedures, a clinical trial has been performed by one of the first users of INVO, the Dr. Elkin Lucena at CECOLFES in Colombia. Dr. Lucena gave us the permission to report the results of the first 125 INVO procedures that he will published in a peer review journal (Table 19.4) [16]. These procedures were done in

Table 19.5 Preliminary results using the INVOcell for oocyte maturation and embryo development after ICSI

Procedure	Countries	Number of cycles	Number of clinical pregnancies (rate)
ICSI	Austria, Brazil, Colombia, Peru, Turkey	190	66
IVM, ICSI	Colombia, Venezuela	7	2
Total		197	68 (34.5%)

female population including 40 years old patients and older with an average age of 33.8. These 120 infertile couples should have been treated with a conventional IVF. Severe male factors needing ICSI were excluded. Mild ovarian stimulation was only used with indomethacin started at day 8 to block the ovulation. An average number of 6.5 oocytes were obtained per punction of which an average of 4.2 was inseminated in INVO. The excess oocytes (2.3 per punction) if matures were cryopreserved for future use.

Potential Disadvantages/Advantages

Potential Disadvantages

Products of degradation from dead cells and metabolism of the live cells are known to be detrimental to fertilization and embryo development. In the INVO procedure, the concentration of motile sperm has been deliberately reduced to 30,000–35,000 motile sperm per milliliter. This concentration represents less than a third of the sperm concentration generally used in conventional IVF. It has been demonstrated that this sperm concentration gives the best fertilization rates, with the lowest production of degradation products and rate of polyspermic embryos (unpublished study). The absence of embryo checking at 16–20 h postinsemination could fail to identify polyspermic embryos. Polyspermic embryos were frequently observed at the beginning of IVF due to an immaturity of the retrieved oocytes and a very high motile sperm concentration used for insemination. The formation of pronuclei is a dynamic process; it seems irrational to try eliminating polyspermic embryos by a few seconds of observation. Eliminate the main causes for polyspermic embryo formation by a better oocyte maturity and by decreasing the sperm concentration has seemed to us a more logical approach.

Advantages

1. INVO dramatically reduces and simplifies gametes and embryos handling and manipulations.
2. An embryologist with little experiences in reproductive technologies can be trained quickly and obtain excellent results.
3. The fixed laboratory equipment is low cost and not complex allowing creation of INVO units requiring little equipment maintenance and quality controls.
4. The frequent electric breakdowns observed in developing countries that affect the results of IVF/ICSI, even when battery backup or generators are available, did not impact the result of INVO.
5. The gametes and embryos are not stored in the laboratory during the 3 days of incubation. Therefore, gas supplementation, battery backup, positive pressure and air filtration, alarm system, and embryologist on call are not necessary. All these factors have contributed to the decreased cost of INVO.
6. The risk of mixing up gametes or embryos has been reduced, and INVO gives the patient a new sense of participation with in vivo conception.

Other Applications of the INVOcell

Gamete and embryo transportation in INVOcell have been reported [9] and are still performed by users of INVO. Reproductive centers have incubators overloaded with eggs and embryos requiring frequent openings of the doors and creating major variations in CO_2 and temperature. The INVOcell has been used successfully to vaginally incubate embryos inseminated through ICSI. The INVOcell has also been used to mature immature oocytes intravaginally and later to incubate the embryos inseminated by ICSI as shown in Table 19.5.

What Did We Learn from INVO?

Oocyte In Vitro Maturation After Retrieval Is Not Required

From the beginning, the oocytes that have being retrieved after hCG induction were immediately inseminated. This was possible by using 36 h between the hCG injection and the oocyte retrieval.

Low Sperm Concentrations Are Used Successfully for Insemination

INVO has always been used with very low sperm concentrations for oocyte insemination. A concentration of 30,000 motile spermatozoa/mL is generally used and gives comparable fertilization rate and pregnancy rates to the conventional IVF. Concentrations as low as 5,000 motile spermatozoa/mL were also used but abandoned due to inconsistency in fertilization (nonpublished).

Embryo Development in Low Oxygen Concentration (≈ 5%)

The INVOcell device is permeable to gas and equilibrates the pCO_2 and pO_2 of the culture medium of the inner chamber with the pCO_2 and pO_2 of the vagina. In this system, the vagina works as a CO_2 generator and an O_2 reducer. The INVO process was the first fertilization system using low oxygen concentration in air.

Cumulus Cells Perform as a Homologous Coculture System

The INVO procedure was the first coculture system using cumulus cells. This coculture system is simple, non-labor intensive, and safest for the patient [17, 18]. These cumulus cells filter and absorb the toxins and nitrogenous residues produced by the dead cells and the metabolism of the live.

Embryo Cumulus Cells Are Removed Naturally During Incubation

Spontaneous removal of embryo cumulus and corona cells has always been observed after 2 or 3 days of incubation in the INVO device. This natural and complete denudation, a sign of quality culture conditions, is the result of the enzymatic action of the spermatozoa as well as a mechanical action related to the patient mobility during the period of incubation. In the INVO procedure, culture media change is not performed so the presence of infection or toxins cannot be tolerated. The oocytes are trapped in a cumulus mass dense and viscous when any toxicity is present during the 2 or 3 days of incubation.

Positioning of INVO Among the Current Reproductive Techniques

The INVO procedure is a new infertility treatment positioned between intrauterine insemination (IUI)—simple, inexpensive, but not very successful (less than 10% of birth per cycle) [19]—and conventional IVF—complex, very expensive, but very successful (28–32% birth per cycle have been reported by several international registries for patient under 40 years old). INVO when associated with mild stimulation is a simple and successful procedure. INVO presents the advantage to treat more indications of infertility than IUI (85 vs. 50%) and to offer a diagnostic value (fertilization or not) that IUI does not.

Potential Role of INVO in Developed Countries

Governmental regulations are in place in many of the developed countries restricting the use of infertility treatments to reproductive centers. These centers have already invested in complex equipment reducing the cost saving of the INVO cycle. However, the simplicity of INVO allows performing three to four INVO cycles for one cycle of IVF or ICSI. Reproductive centers can treat a lot more patient without overloading incubators or investing in new equipment and increasing the number of embryologists. INVO may also be used in satellite units to extend the geographic influence of the reproductive center.

Role of INVO in Developing Countries

It seems irrational to treat infertility in developing countries that are frequently overpopulated, have difficulties in feeding their existing population, and have other medical priorities than to treat their infertile population. However, infertility has major socioeconomical consequences in developing countries [20, 21]. Couples who do not have children are rejected socially and do not provide offspring that economically support the elderly population. It is estimated that by 2050 most of the developing countries will not be able to renew their population [21]. The main cause of infertility is blocked tubes due to infectious diseases. Unfortunately, IVF or ICSI, the most effective treatments, is not available due to the cost to build a reproductive center and the cost of a treatment cycle. The low cost of equipment used in an INVO unit, the low maintenance of this equipment, and the rapid training of an embryologist with little experience are factors which will contribute to the expansion of the role of INVO in the treatment in infertility in developing countries.

Future Implications and Developments of the INVO Technology

Only a low percentage of infertile couples in the world can benefit from the treatment of assisted reproduction techniques due to their cost and availability.

If the cost per pregnancy using INVO confirms to be lower than the cost per pregnancy using IUI, INVO should replace IUI as the first treatment option for infertility. The diagnostic value of INVO and its ability to treat a larger percentage of the infertile population than IUI are other contributing factors for using INVO as the first-line treatment of an infertile couple.

In developing countries or regions of the world where IVF/ICSI is not available or is not affordable for a large majority of infertile couples, INVO combined with natural cycle or mild stimulation and performed in INVO satellite units represents an attractive infertility treatment option. These INVO units can be built very rapidly and do not need a large financial investment in fixed equipment. The maintenance does not require a lot of expenses and need little quality controls. The units can work even when gas supplies and

electric power are erratic. The trainings of the clinician and the embryologist can be completed rapidly. The INVO procedure is so simple that the results are highly reproducible even with an embryologist with little experience. INVO involves the participation of the patient in the process of fertilization and early embryo development. INVO is an in vivo conception with a high level of acceptance among the patients who benefited from the procedure especially patients with religious convictions and ethical concerns for conventional IVF.

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Microfluidics for Gamete Manipulation and Embryo Culture

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Gary D. Smith, Charles Bormann, and Shuichi Takayama

Abstract

Microfluidics is an emerging field that holds immense potential for scientific discovery. By utilizing microfluidics, knowledge can be obtained in basic gamete/embryo developmental biology as well as expand our understanding in specialized areas, such as assisted reproduction. This review describes the technology of microfluidics and discusses applications for assisted reproduction technology. Development of an integrated microfluidic platform for assisted reproduction, which can manipulate gametes, embryos, their culture environment, and incorporate biomarker analysis, could have a dramatic impact on basic understanding of embryo physiology as well as provide significant improvements in current assisted reproductive technologies focused on treating infertility and preserving fertility.

Keywords

Microfluidics for gamete manipulation • Embryo culture and microfluidics • Biomarker analysis in IVF

Assisted reproductive technologies (ART) have evolved over the last 3 decades with the development of new technologies that have resulted in refinement of in vitro production of mammalian embryos which is central to several other areas of biotechnology. In vitro production of mammalian embryos is utilized for treatment of human infertility [1], derivation of genetically modified animals for biomedical research and enhanced food production [2], cryopreservation of gametes, and nuclear transfer [3]. Continuous refinements of ARTs have been achieved through modification of medium components, their concentrations, and supplementation of new components at specific time points. While there have been many advances in IVP in regard to efficiency and embryo quality, these embryos are still suboptimal compared to their in vivo counterparts. Differences in the quality of embryos produced in vitro vs. in vivo are believed to result from stresses imposed on oocytes/embryos during the in vitro production procedure. These stresses begin as early as oocyte retrieval, as even a small variations in handling temperature affects ability of oocytes to continue through the in vitro production procedure [4]. Additionally, ART procedures entail as numerous washes and transfers to different culture drops that potentially impose stress (change in pH, osmolarity, and temperature) on gametes and embryos [5]. Finally, human factors associated with ARTs have potential to introduce oocyte/embryo loss at each handling step. In treatment of human infertility, where costs of embryos are high, loss of an oocyte or embryo at any stage could be problematic. Historically, scientists have worked to develop tools that would allow gamete/embryo manipulation under more in

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vivo-like conditions, with the idea that this would reduce stress imposed during in vitro production and potential human error. Today, significant developments in fabrication of micro-/nanotechnology devices are positioned to impact biology and medicine and have the potential to significantly improve laboratory ARTs for humans infertility treatment, domestic animals food production, rodents biomedical research, and stem cell research and the search for treatments and cures for human health diseases.

Integration of microdevices into biological research and medical treatments has progressed rapidly [6–9]. Developments of micro-/nanodevices have enabled the study of cells at single-cell and subcellular levels in ways not previously possible. These devices may allow us the ability to track protein production, metabolism, as well as additional viability markers in living cells vs. current fixed cell systems. These types of tools become especially important when the availability of cells is limited, such as when dealing with gametes and embryos in treating human infertility. In addition, microfluidic devices could allow development of more in vivo-like systems for cell culture [10] and embryo development [11] by precise environmental control at the microscale.

Microdevice and Microfluidic Technologies

Advancements in nanotechnology and miniaturization of bioanalytical procedures have allowed scientists a powerful platform to study biological systems. Deoxyribonucleic acid (DNA) sequencing, PCR, electrophoresis, DNA separation, and cell counting and sorting are just a small list of biological systems that have begun transformation from the macro- to microscale [9]. Microfluidics is one area of advancement with great promise. Microfluidics deals with the behavior, specific control, and manipulation of microliter and nanoliter volumes of fluid. This technology first emerged in the 1990s and has enabled molecular biology procedures and single cell-based bioassays. One example of how this technology has been applied was in development of a polydimethylsiloxane (PDMS)-based microfluidic device for the analysis of a single rat pheochromocytoma (PC12) cell. This system maintained cell viability and could be utilized to transport a single PC12 cell into a microchamber where nicotine was introduced and microelectrodes were used to effectively monitor release of dopamine from the cell [12].

Microfluidic systems have been designed for a unidirectional flow of oocytes, embryos, sperm, medium, and medium components [6, 13–16]. In some instances, microscale design of these microfluidic devices permits laminar flow, characterized as parallel streams flowing without disruption between currents. When laminar flow is present, mixing between streams occurs predominately by diffusion [9] with a slight meniscus between the laminar flowing streams. Similar methodology could be used to spatially and temporally expose gametes, embryos, and embryonic stem cells to different molecules with subcellular precision. In addition, one could develop molecular gradients to investigate migratory events of gametes, embryos, and stem cells. The unique physical, spatial, and temporal environment obtained with microdevices opens countless avenues to increase knowledge of basic gamete/embryo physiology that were not previously possible.

Assisted Reproductive Technologies

Currently, in vitro production of mammalian embryos is relatively inefficient due to suboptimal starting materials and artificial stresses imposed on gametes and embryos in their in vitro environment. During the laboratory ART process, oocytes/embryos are moved numerous times between media, requiring removal from their incubator environment. These manipulations introduce stresses such as changes in temperature, pH, osmolarity, and mechanical stress. Past efforts to reduce biochemical stress to gametes and embryos have involved manipulation of media and its composition. Relatively little attention has been paid to improving the mechanical platform on which gamete/embryo manipulation occurs.

In Vitro Oocyte Maturation

Oocyte maturation in vitro is the first step of in vitro production of domestic animal embryos. While human oocytes are usually obtained at mature state following in vivo maturation, there are groups working on human oocyte in vitro maturation (IVM) [17–20]. Oocyte maturation can be divided into two independent events: nuclear maturation (progression to metaphase II [MII]) and cytoplasmic maturation (preparation of the cytoplasm to support development to MII, subsequent fertilization, and embryo development). Given that embryo viability closely reflects oocyte quality, it is clear that optimal oocyte maturation is essential for successful preimplantation development.

Oocyte IVM is an emerging technology that holds great potential for treatment of human infertility (see reviews [17–20]). This technology, once efficient, will reduce the need for ovarian hormone stimulation, limiting the risk of ovarian hyperstimulation (OHSS) and reducing the cost associated with in vitro fertilization (IVF) [21]. Additionally, oocyte IVM may provide an alternative to cancelation of treatment cycles for patients with a poor response to ovarian stimulation [22, 23]. Finally, IVM may be a useful tool coupled with oocyte, follicle, or ovarian tissue cryopreservation

for preserving fertility in women undergoing gonadotoxic chemo- or radiation therapy for cancers and/or autoimmune diseases. Today, human IVM is relatively inefficient with many questions regarding safety of this technique [24–26]. Thus, it is imperative to develop a culture system that adequately supports nuclear and cytoplasmic maturation of oocytes.

It can be hypothesized that oocyte IVM in a microfluidic environment more closely resembles oocyte maturation conditions in vivo and thus may benefit oocyte maturation and subsequent embryo development. This is especially important as it has been shown that events during oocyte maturation affect subsequent embryonic development [27–29]. Walters and colleagues reported that, despite inferior cumulus expansion, PDMS microchannels did not inhibit porcine oocyte nuclear maturation (71% compared to control cultured oocytes 61%) [30]. Hester and coworkers subsequently demonstrated a significant increase ($P<0.05$) in embryos cleaved from total oocytes matured in PDMS/borosilicate microchannels (67%) compared to controls (49%) [31]. This improvement in embryo development was speculated to result from improved cytoplasmic maturation. These initial experiments on oocyte maturation using microfluidics are promising and may provide a more supportive in vivo-like culture environment for maturing human oocytes in the future.

Sperm Selection and Isolation

Nearly 40% of human infertility is male factor-related [32]. IVF was originally developed as a treatment for tubal infertility; however, high rates of males with poor semen quality have led to development of several methods to isolate motile sperm. Under in vivo conditions, potentially fertile spermatozoa are separated from immotile spermatozoa, debris, and seminal plasma in the female genital tract [33]. The three most commonly used techniques to artificially isolate motile sperm from an ejaculate include density gradient centrifugation, sperm migration or "swim-up," and simple dilution and washing [34]. Both sperm migration and gradient centrifugation result in recovery of motile sperm; yet, several investigators have raised concern as to whether these methods of sperm isolation damage sperm DNA or impair motile sperm by exposing them to reactive oxygen species [35–37]. It has been shown that increased levels of sperm DNA damage during IVF correlate with reduction in embryo morphology at early cleavage stages [38], failure to advance to the blastocyst stage in vitro [39, 40], decreased pregnancy rates [41–43], and result in an increase in spontaneous abortions [44]. Furthermore, these separation techniques are suboptimal for patients with severe oligozoospermia, as samples from many of these patients have large amounts of debris, and recovery rates from initial sperm samples have been reported to be as low as 0.8% for direct sperm migration [45, 46].

Reduced spermatozoa quality or quantity during IVF results in poor and sometimes failed fertilization. In these cases, fertilization by injecting a single spermatozoon into the ooplasm of a mature oocyte is possible and referred to as intracytoplasmic sperm injection (ICSI) [47]. Although only a single healthy sperm is required for ICSI, processing, isolating, and selecting the most viable sperm can be challenging with current sperm separation techniques. Importantly, one can recognize that ICSI is an invasive technique and circumvents processes of natural fertilization [48]. Because of its invasiveness, even in fully competent hands, this technique can result in lysis and death of 5–7% of injected oocytes [49, 50]. Considering that ICSI bypasses all normal sperm selection processes, it is imperative to have an efficient artificial selection process that minimizes stress and damage to sperm and maximizes the probability of successful pregnancy, birth, and healthy offspring [48]. Therefore, separation of sperm using microfluidics has been investigated as a possible alternative to traditional sperm isolation techniques.

Advances in microfabrication have made it possible to easily construct any desired [51, 52] microstructures. Using soft lithography, Cho and colleagues developed a novel gravity-driven pumping system to sort sperm samples [13]. The device, termed a microscale integrated sperm sorter (MISS), contained inlet/outlet ports, fluid reservoirs, gravity-driven power sources, and converging microchannels with laminar flow; all integrated components working together to facilitate sperm sorting (Fig. 20.1) [13]. The premise for this design was based on a natural phenomenon of parallel laminar flow, characteristic of fluid movement at the microscale [16]. The orientation, geometry, and size of the MISS reservoirs were designed to balance gravitational forces and surface tension forces and provide a pumping system, free of external energy, that generates steady flow rates over extended periods of time [13]. This device was designed so that converging stream of semen and media would flow in parallel, in a laminar fashion within a microchannel. The two parallel streams only mix by diffusion at the interface between streams, but motile sperm are able to swim across the contacting streamline and into the media for collection. Nonmotile sperm, cellular debris, and seminal plasma do not cross this barrier and flow uninterrupted into a waste reservoir.

For unprocessed semen, the device consistently produced a sorted fraction with increased motility (mean 98% motile) and improved strict sperm morphology (mean 22% normal forms) vs. the initial specimen (mean 44 and 10%, respectively) [16]. For debris-filled samples, the device not only concentrated motile sperm (mean 98% motile) within the collected fraction but also produced a round cell: sperm ratio of 1:33 compared with a 10:1 ratio in the starting specimen [16].

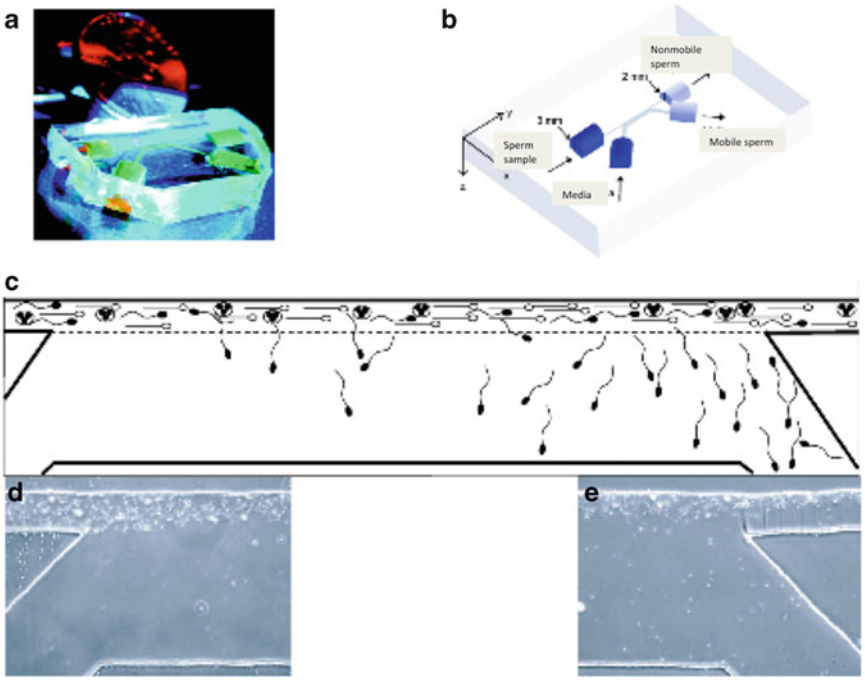

Fig. 20.1 (**a**) Picture of microfluidic sperm sorter next to a penny for size reference. (**b**) Three-dimensional view of microfluidic sperm sorter. (**c**) Theoretical vision for microfluidic sperm sorter. Media and semen flows from left to right. Semen sample is loaded into the upper stream inlet, and fresh media is placed in the lower stream inlet. (**d**, **e**) Motile sperm deviate from the initial streamline and cross the interface of the laminar flow streams, exiting into the lower stream outlet for recovery. Debris, nonmotile sperm, and seminal plasma exit the device in the upper stream outlet. From Cho et al. [13]. Used with permission

This device provides a simple, safe method of obtaining motile sperm of enriched normal morphology from both unprocessed normal semen and poor-quality specimens containing significant debris.

A limitation of the current microfluidic device is that it is not capable of processing an entire sample due to its small capacity, yet such limitations can be circumvented by parallel processing. Additionally, it does not isolate every motile sperm, but this again can be elevated, if need be, by integrating a semen recirculatory system to current chip designs. Nevertheless, isolation of motile sperm is fast and easy and can be readily used for isolating sperm for ICSI, for insemination in microdrops under oil, or for insemination in a microfluidic environment. Because no centrifugation is employed in MISS-sperm isolation, there is reduced opportunity to cause sublethal structural or DNA damage to sperm that may be used for insemination. Additional modifications of the current system are underway, which may allow for large-scale processing of semen.

In Vitro Fertilization

Species differences exist in the relative difficulty in achieving IVF. This is especially apparent in animal systems, such as the pig, where polyspermy occurs at a high frequency and lab-to-lab variations are seen in the blastocyst formation (25–30%). In general, IVF involves coincubation of oocytes with an appropriate concentration of motile and morphologically normal sperm. Media volumes may range from 20 mL microdrops to 1 mL in dishes at a minimum concentration of 500,000 sperm/mL [34]. The volume of media in which insemination occurs varies between laboratories and between total numbers of motile sperm collected from an individual. Because traditional insemination in a microdrop requires such a high number of sperm which then deplete metabolic substrates and produce potentially harmful waste products in the IVF media [53, 54], there is an emphasis to develop new technologies for in vitro insemination to reduce numbers of sperm needed to achieve successful IVF.

Several systems have been developed for insemination, including the climbing-over-a-wall (COW) method used by Funahashi and Nagai and straw IVF [55, 56]. The COW method has been performed with frozen-thawed boar sperm placed into an outer chamber filled with tris-buffered medium for fertilization of porcine oocytes positioned in the inner well of the COW chamber. This system was designed to select high-quality sperm in terms of progressive motility and viability and potentially mimics the in vivo system of the reproductive tract. The COW-IVF method resulted in high penetration rates at increased sperm concentrations of 0.5, 1.0, and 5.0×10^5 cells/mL while still maintaining monospermic penetration rates comparable to the traditional IVF microdrop system [55]. Similarly, the straw IVF method was developed to reduce the incidence of polyspermy and maintain penetration rates [56] and utilizes a 0.25-mL straws with oocytes in medium (~5 cm length) at one end of the straw and an adjacent 1-cm section of medium containing

spermatozoa. Thus, sperm must swim to the oocytes in order to initiate fertilization, and this method reduced the number of sperm at the site of fertilization by selecting the highest quality sperm [56]. Both the COW and straw IVF methods limit the total number of sperm reaching the egg, enriching the population for those with the best motility. Similar methodologies have also been applied to human IVF with some success [57–59]. However, these techniques have not gained widespread acceptance.

Advancements in microfluidics and soft lithography have made it possible to more closely mimic the oviductal (site of in vivo fertilization) environment in vitro. Research by Clark and colleagues studied porcine fertilization in a microchannel designed to recapitulate function and structure of the oviduct [60]. In this study, oocytes in microchannels had a significantly higher incidence of monospermic penetration ($P < 0.05$) as compared to oocytes fertilized in a traditional microdrop system, with comparable penetration and male pronuclear formation rates [60]. These results demonstrate that the biomimetic microchannel IVF system can reduce polyspermy and thereby increase number of potentially viable embryos without reducing overall in vitro production efficiency.

It has been well demonstrated that only a few hundred sperm eventually reach the ampulla of the oviduct for fertilization in humans [61, 62]. Advancements in microchannel design and microfluidics may ultimately provide an alternative method of in vivo-like insemination by decreasing volume of media and numbers of sperm needed, while increasing oocyte-sperm interaction in a dynamic environment. Suh and colleagues demonstrated that mouse IVF can be conducted quickly and successfully within microfluidic channels [63]. In this experiment, a PDMS-based microfluidic insemination channel was fabricated consisting of a single microchannel separated by a barrier grate manufactured in three dimensions (Fig. 20.2). This grate permits unimpeded flow of sperm and media through the microchannel but prevents migration of the oocyte [63]. Fertilization rates were significantly increased in microchannels, compared to conventional means of insemination, when the concentration of sperm used for insemination was limited [63]. Not only were less sperm required for fertilization because of the small amounts of media needed for microfluidic culture, but also a lower concentration of sperm was required because of constrained space and direct delivery of sperm to the oocyte, further reducing the number of sperm needed for successful fertilization [63]. The use of microfluidics and flow results in a predictable delivery of sperm to oocytes and may reduce the randomness of sperm and oocyte interaction that takes place in larger volumes during standard IVF [63]. Finally, this method may be useful when inseminating with sexed semen and/or [47] samples with compromised sperm numbers due to cryopreservation and thawing.

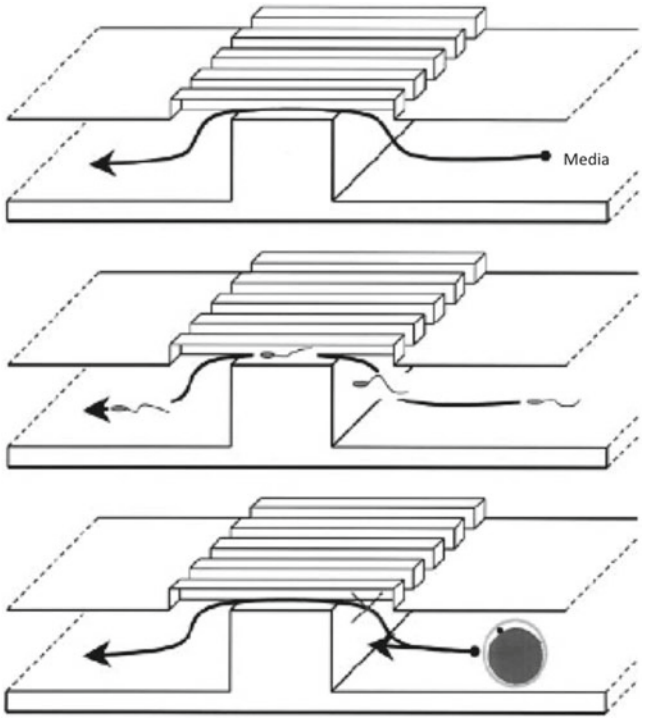

Fig. 20.2 Schematic of a microfluidic device composed of a microchannel for coincubation of sperm and oocytes for IVF. The three-dimensional construct of the channel prevents movement of the oocyte beyond the grate but allows for flow of medium and sperm through the channel. The potential to recirculate media and sperm exists. From Suh et al. [63]. Used with permission

Embryo Culture

In vivo preimplantation mammalian embryos develop in the absence of direct cell contact with the reproductive tract for approximately 5–7 days before implanting in the uterus. These embryos are free floating, lack a blood supply, and move continuously within the female reproductive tract through a changing fluid environment. During this time, the embryo is undergoing mitotic divisions and differentiation. Preimplantation embryo's nutrition is derived from internal stores or maternal-derived products obtained from luminal secretions of the oviduct and uterus. Its cellular activities, including division, gene expression, and metabolism, are influenced by the environment and factors contained within the embryo, produced by the embryo itself, and produced by cells of the reproductive tract.

Success of IVF may be compromised by limitations of current embryo culture methods, resulting in impaired embryo development and decreased viability [34]. Improvements in embryo culture methods have been based on simulating in vivo conditions and meeting the ever-changing needs of the embryo. Research into sequential media methods of embryo culture takes this hypothesis one step

further [64]. Conventional embryo culture systems, however, mostly employ a single medium, or double media system, from the time of insemination until embryo transfer. In vivo, the embryo is bathed in a constantly changing environment as it moves through the oviduct to the uterus. Conditions required by the embryo at one point of development may be detrimental at later stages.

Current embryo culture technologies are not ideal for sequential media embryo culture systems. Embryos must be handled during media changes, and environments are voluminous compared to in vivo conditions which may temper the presence of embryotrophic factors. Microfluidics is well suited to meet the needs of embryo culture. Changing of media is straightforward, and no manipulation of the embryo is necessary. The media can be gradually changed around the embryo, rather than subjecting it to sudden changes in microenvironment. In theory, microenvironment that exists within microfluidic devices might simulate physiological conditions and therefore also may benefit embryo development [6].

Thus far, there have been encouraging outcomes with microfluidic embryo culture technology. Glasgow and coworkers first established that manipulation and movement of an embryo in a microfluidic environment are possible at low flow rates. In this experiment, microchannels were tested and designed with constriction areas that prevented movement of the embryo down the channel while allowing for flow of media around and past the embryo. No deformation or injury to the embryo was noted at the low flow rates used [65]. Once the feasibility of embryo handling and media flow within a microchannel was established, mouse embryos were cultured and development rates compared [6]. A significantly higher percentage of embryos within the microfluidic channels reached the morula, blastocyst, and hatched blastocyst stage compared with controls. In addition, early embryonic development was investigated to determine if PDMS/borosilicate microchannels under static conditions could support development of in vivo-derived four-cell porcine embryos to blastocysts. There was no difference ($P>0.05$) in the blastocyst formation in microchannels (79%) compared to control drops (84%) [66]. In addition, some microchannel-produced blastocysts were transferred to a recipient and produced five live piglets with normal birth and weaning weights and subsequently went on to reproduce normally [66].

In 2004, experiments were conducted to determine if microchannel culture could support development of two-cell mouse embryos. Using PDMS/borosilicate glass microchannels, it was reported that there were significantly more ($P>0.01$) blastocysts in the microchannels than in controls following 72 h of culture (73, 43%, respectively) [67]. Using another prototype (silicon wafer, borosilicate sandwich), it was demonstrated that percentage of blastocysts were also increased compared to control and the kinetics of development was more in vivo-like. Birth of live offspring was achieved following culture in microchannels [66].

Similarly, Heo and coworkers demonstrated an advantage of culturing mouse embryos on a microfluidic platform with dynamic media flow [68]. These experiments utilized a computer-controlled, integrated microfluidic system with on-chip pumps and valves, powered by individually actuated Braille pins to pump fresh media through a series of elastomeric capillaries [69]. This system takes advantage of the resilient yet elastic nature of PDMS microchannels fabricated with soft lithography, together with the movement of Braille pins to "squeeze" fluid through channels. When synchronized to various valving patterns, each stroke of a Braille pin generates forward or backward flow of media through microchannels. Results from these experiments showed embryos cultured under dynamic conditions had significantly more cells than those cultured under static conditions. Furthermore, blastocyst cell counts following microfluidic dynamic embryo culture more closely mirrored results obtained from their in vivo counterparts [68]. These results suggest that development of embryos in a dynamic microenvironment may closely mimic in vivo embryo growth conditions. In addition, these improved embryo development rates obtained in microfluidic dynamic culture experimentally translated into improved implantation and ongoing pregnancy rates. Most importantly, these preimplantation embryo developmental kinetics, implantation, and ongoing pregnancy rates more closely resembled those observed when embryos developed in utero in comparison to embryos grown under conventional static conditions. Live offspring were also produced. Collectively, results from these comparative controlled studies suggest that the microenvironment obtained by combining microfluidics, dynamic pulsatile fluid flow, and microfunnel embryo detainment supports enhanced embryo development compared to conventional culture conditions. One could question whether this improved embryo development was a consequence of suboptimal development of control embryos. It is impossible to compare results from different laboratories, strains of mice, initial stage of embryo development, soluble culture components, gaseous environments, timing of end-point measures, and specifics of end-point measures. However, our advanced blastocyst development rates, total blastocyst cell count, implantation rates, and ongoing pregnancy rates for controls are representative of contemporary reports [70–75]. Using the same culture device design, Bormann and coworkers (unpublished data) showed a significant improvement in percentage of IVF bovine embryos reaching the blastocyst stage compared to static controls. These results further demonstrate the importance of physical and mechanical environment on embryo development as well as the potential wide application of this technology in gamete/embryo culture.

To begin elucidation of mechanisms imparting improved embryonic developmental competence in pulsatile dynamic microfluidic culture, we ask the question of whether fluid flow influenced embryo development in a temporal- or developmental-stage-specific manner. We found that

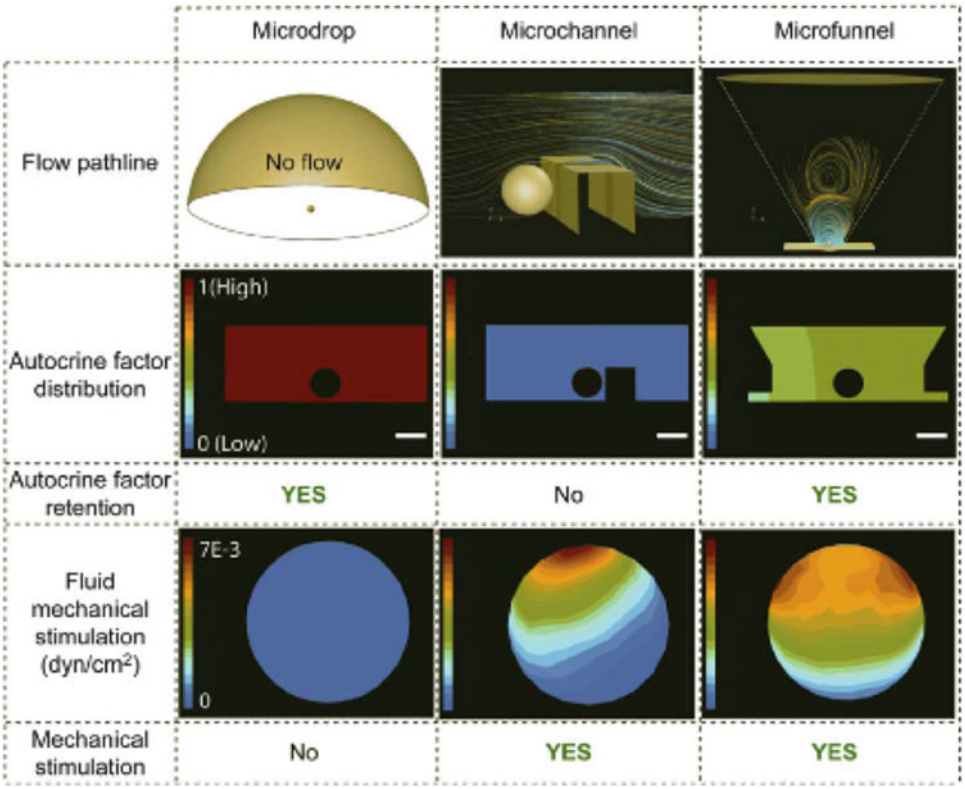

Fig. 20.3 Dynamic microfunnel culture provides fluid mechanical stimulation with retention of autocrine factors. Summary of flow path lines, mechanical stimulation, and autocrine factor distribution/retention provided by different embryo culture methods as simulated using Fluent. Microdrops retain autocrine factors but do not provide mechanical stimulation. Microchannels provide mechanical stimulation but do not retain autocrine factors. Dynamic microfunnel cultures provide both. From Heo et al. [68]. Used with permission

developmental impact of pulsatile media flow is not developmental stage specific [68]. This is especially important when one considers that mouse zygotic genome activation occurs at the one- to two-cell transition [76] during the first 48 h of culture, in conjunction with global chromatin remodeling, altered histone acetylation, and release from a transcriptionally repressive state [77]. The lack of differential benefit of pulsatile media flow between the first and last 48 h of culture would also suggest that the developmental benefit is not specific toward latter preimplantation developmental events such as compaction at the eight-cell stage [78], initiation of cellular polarity at the eight-cell to morula stage [79], or blastocoel formation at the transition form morula to blastocyst [80]. These results would suggest that benefits of microfluidic-generated pulsatile fluid flow on embryonic developmental competence may be due to subtle changes in the culture microenvironment that produces embryo development that is more in vivo-like and significantly advanced compared to static culture. This leads one to consider dynamic culture, embryo development, and microenvironment in relation to retention of autocrine factors, removal of waste products, and disruption of concentration gradients that may form at cell surfaces.

Embryos may develop better in small volumes or in the presence of multiple embryos, presumably due to beneficial autocrine effects [81, 82]. Some identified autocrine factors include leukemia inhibitory factor [83], interleukin-1 [84], insulin-like growth factor [85], platelet-derived growth factor [86], epidermal growth factor [87], and transforming growth factor [88]. Utilization of embryo-secreted autocrine factors is advantageous over exogenous delivery of mitogens because (i) embryos produce a complex mixture of biochemicals which are difficult to completely mimic with exogenous biomolecules and (ii) addition of exogenous growth factors has inherent limitations in spatial and temporal exposure and can sometimes lead to developmental abnormalities such as large offspring syndrome (LOS) [89, 90]. Heo and coworkers employed a computational modeling system (Fig. 20.3) to simulate fluid flow and biomolecular transport within the microfunnel culture systems. The results show that the microfunnel culture systems retain greater amounts of biomolecules compared to microchannels with flow, which had suboptimal embryo development [91]. However, retention of beneficial autocrine factors cannot fully explain the benefits of microfunnel pulsatile culture since this occurs to a greater extent in microdrops, which displayed reduced

embryo developmental competence compared to the microfunnel dynamic system.

Embryos culture in small droplets may allow accumulation of toxic substances such as ammonia- [92] and oxygen-derived radicals [93], which may harm embryos [75]. Periodic media changes could prevent toxin accumulation, but it needs to be balanced with elimination of positive-acting auto- and paracrine factors [94]. The microfunnel pulsatile embryo culture system provided periodic media refresh, moderate retention of biomolecules, agitation of fluid surrounding the embryo, and removal of media. This combination appears to be advantageous.

Another concept to acknowledge within a microfluidic/microfunnel/dynamic culture system is the inherent fluid movement and mechanical agitation of embryos during culture that can disrupt concentration gradients of substrates, secretory molecules, dissolved gases, and waste products. Movement ensures that unstirred layers do not form around the embryo and likely facilitate exchange of gases and/or metabolites. While such point-of-contact concentrations of gases, substrates, or metabolites are difficult to measure, one can envision that such disruptions of gradients could occur with media flow and embryo agitation. In conclusion, a portable computerized microfluidic system has been designed for embryo culture. Its user-friendly architecture, flexibility in microchannel and chip design, and programmability of fluid actuation system allow convenient and practical manipulation of chemical and mechanical microenvironments for in vitro embryo production. Improved pregnancy outcomes may alleviate current inefficiencies in embryo production for biomedical research, genetic gain in domestic species, and fertility treatment in humans. Combining physiological mechanical stimulation with retention of beneficial autocrine factors may also benefit a broad range of cell culture technologies beyond the dynamic microfunnel embryo culture systems described here.

Integrated ART Microfluidic Device

A significant advantage of using microfluidics is in the ability to integrate various aspects of ART and cell monitoring systems on a single chip. Clark and colleagues reported the integration of two different techniques of ART (maturation and fertilization) in a single microchannel with porcine oocytes [95]. Additional research is needed in design and development of a platform that can meet all the needs of ARTs on a single chip. This will not only mirror more closely the maternal microenvironment but will also eliminate technician-dependent variability and error.

Integration of detection devices with somatic cell culture has already been achieved using microfluidics. Cheng and colleagues demonstrated the ability to monitor Ca^{2+} transients, extracellular pH, and relative amounts of intra- and extracellular lactate production from a single cardiomyocyte [96]. Furthermore, Shackman and colleagues developed a novel system for high-resolution monitoring of insulin secretion from single islets of Langerhans [97], and Mehta and colleagues developed a method to measure oxygen content in real time in microfluidic perfusion systems [98]. Such assays, if applied to a microfluidic platform for gamete/embryo culture and analysis, could function as a monitoring system for culture condition optimization and real-time selection of embryos with the greatest implantation potential.

Embryo Bioanalysis

An unmet need in the infertility laboratory is selecting the most viable embryo for transfer from a pool of visually similar embryos. Accepted practice in most human clinics is to transfer embryos either on day 3 (approximately the eight-cell stage) or on days 5 and 6 (blastocyst stages). It is difficult to predict which day 3 embryos will continue to develop to the blastocyst stage, and importantly, this developmental stage coincides with the maternal to embryonic genomic transition in which a high percentage of embryos undergo developmental arrest. Therefore, many programs transfer multiple embryos to obtain acceptable pregnancy rates. Appropriate efforts currently focus on reducing the number of multiple pregnancies by move toward single blastocyst transfer [99], making embryo selection even more critical. In the past and present, embryo selection was/is based primarily on morphological characteristics such as cleavage rate, cell number, blastomere size, and degree of fragmentation. These methods of selection are subjective and lack predictive power.

One of the most reliable indicators of embryo viability is the rate at which an embryo consumes glucose or pyruvate from the culture medium and produces lactate and other metabolic waste products. Lane and Gardner showed embryos with glycolytic activity similar to their in vivo counterparts have a fourfold increase in pregnancy rate compared to embryos selected and transferred at random [100]. Recently, Bormann and colleagues (unpublished data) developed a metabolic assay that can be readily integrated into a microfluidic platform. This will allow noninvasive quantification of metabolism by individual preimplantation embryo [101]. This assay along with others currently being developed may soon be used to assess embryo quality and viability.

Advancements in proteomics have made it possible to identify minute quantities of secreted peptides from preimplantation stage embryos. Using microliquid chromatography electrospray ionization mass spectrometry and matrix-assisted laser desorption ionization time-of-flight mass spectrometry, Katz-Jaffe and coworkers analyzed the proteome of individual human embryos and related protein

expression profiles to morphology [102, 103]. Protein profiling has many benefits and potential applications in assisted reproduction. By measuring an embryo's secretome, it may be possible in the future to identify certain markers for male and female embryos and determine the sex of the developing embryo without using invasive approaches. This may be an important diagnostic tool in circumventing X-linked congenital disorders. Furthermore, another benefit to this noninvasive approach to profiling developing embryos would be to detect embryos with chromosome abnormalities or other health issues. Further advances in protein profiling would not just enable selection of the best quality embryo but may also allow identification of abnormal embryos without having to perform invasive procedures such as embryo biopsy and preimplantation genetic screening.

Conclusions and Future Directions

Upon recent developments of microscaled systems similar in size to mammalian gametes and preimplantation embryos, the birth of a new era has begun for assisted reproduction. Important functions necessary for carrying out basic ART laboratory procedures, including oocyte maturation, insemination, and embryo culture, can be performed in microfluidic devices. The idea of integration of numerous techniques into an "ART lab on a chip" is now possible and will allow for automation and chemical and mechanical manipulation. Microfluidic platforms may provide insight as to why ART-produced embryos are suboptimal compared to those derived in vivo. Such platforms also will provide general information to improve our knowledge of basic gamete/embryo physiology and developmental biology. Lastly, new prototypes of microfluidic devices for ARTs will be developed that enable real-time live oocyte/embryo metabolic and secretome analysis, without human manipulation-introduced error. Such live-cell assays will enhance our understanding of cellular physiological processes and provide additional parameters to measure oocyte/embryo health and viability.

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Part V

Sperm Processing and Selection

Sperm Assessment: Traditional Approaches and Their Indicative Value

21

Margot Flint, Fanuel Lampiao, Ashok Agarwal, and Stefan S. du Plessis

Abstract

The traditional semen analysis is the established cornerstone of assessing male fertility, and the diagnostic management depends on a sequential, multi-step approach. Recognized reference values for normality are essential due to the relationship between sperm quality and fertility. The information provided by a semen analysis is the least invasive and most cost-effective assessment of a male's fertility status. Despite the introduction of alternative techniques such as computer-assisted sperm analysis and the advancement of assisted conception, the prediction of fertilization in vitro is still crucial.

Keywords

Sperm assessment • Male fertility • Computer-assisted sperm analysis

The basic semen analysis plays a pivotal role in the diagnosis of male infertility and makes a significant contribution to the diagnostic process in andrology, gynaecology and clinical urology [1]. In 1902, the man considered to be "the founding father of modern andrology", Edward Martin, proposed that an analysis of a semen sample should be incorporated into all infertility assessments [2, 3]. Following this suggestion, in 1956, the scientist John MacLeod advanced the basic semen analysis from beyond a mere observation and introduced the importance of certain sperm parameters such as morphology and motility [2, 3].

The present day examination includes the analysis of certain established semen parameters which can provide key information about the quality of a patient's semen and the functional competence of the spermatozoa [1]. A semen analysis including sperm assessment is a valuable diagnostic tool in assessing possible disorders of the male genital tract and the secretory pattern of the male accessory sex glands. This information can help to determine the reproductive capacity of the male and can be used in conjunction with the partner to indicate the impact of male genital pathophysiology in the assessment of a couple's prospect for fertility. The World Health Organization (WHO) has provided both reference values, based on the semen of fertile men, and a multi-step manual for a semen analysis. The traditional analysis is essentially performed in three functional steps: pre-analytic, analytic, and post-analytic phases [4, 5]. The WHO manual is generally accepted as the standard reference that establishes uniformity in laboratory procedures [6]. It is furthermore useful in providing essential ranges and lower limits of normality from which prognosis of fertility or diagnosis of infertility can be extrapolated by the clinician [7]. The parameters outlined by the WHO include the following: assessment of sperm characteristics and the physical and biochemical analysis of semen [1]. The reference values provided by the latest WHO manual are based on retrospective and prospective analysis of semen samples from hundreds of males from different countries whose partners had a time-to-pregnancy (TTP) of less than 12 months [8, 9].

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Semen parameters can vary greatly among individuals; therefore, laboratories should interpret these result values together with clinical information of the patient. Interpretations of results are also challenging as different laboratories base their analysis on their own particular "reference values", and hence, lack of standardization is often a dilemma [7]. With regard to assisted reproductive technology (ART), semen parameters from analyses that fall into the 95% confidence interval (CI) are not necessarily guarantees of fertility [8]. A basic semen analysis can show diverse irregularities or deviations from the standard reference values. A follow-up investigation can then be carried out in order to assess specific sperm functions and prepare a diagnosis.

Despite the relevance of a semen analysis, it still has limitations in its diagnostic potential. It must be remembered that semen is naturally heterogeneous, and the composition is influenced by several variables, for example, infection, accessory sex gland functioning and the period of sexual abstinence [6]. All of these confounding elements can result in deviation from the standard reference values of the semen parameters and can subsequently alter their indicative value. The limitations of analyses have been described as the problem that it is "a visual observation of a continually variable biological product" [6]. It must be considered that there is no strict feedback system that controls the composition of semen. Consequently, variation may occur among individuals, countries and even between consecutive samples obtained from the same patient [6, 9]. Certain factors are also responsible for potentially altering the results of a semen analysis, such as the time period from collection to analysis [10]. The traditional semen analysis is an extremely valuable laboratory procedure. However, the crucial step is remembering that each variable alone is neither a powerful sole discriminator nor a predictor of fertility status and must therefore be considered in the context of other variables [11]. While measurements made on the whole population of ejaculated spermatozoa cannot define the fertilizing capacity of the few spermatozoa that reach the site of fertilization, the semen analysis nevertheless provides essential information on the clinical status of the individual [8].

It must also be noted that it is impossible to characterize a man's semen quality from evaluation of a single semen sample. It is therefore advisable to examine at least two or three samples before making any conclusions.

The Standard Semen Analysis

The traditional spermiogram is a test which can provide information on several levels. It can assess whether or not a patient is to be considered "normal" based on the quality of his semen and whether or not the degree of this impairment would compromise his fertility status [12]. Secondly, the test can pinpoint an abnormality such as teratozoospermia and can therefore offer a diagnosis [12]. Despite the option of computer-aided semen analysis (CASA), a semi-automated technique, the manual analysis of semen parameters is still an extremely effective indicator of the quality and composition of semen [11].

Table 21.1 The different macroscopic and microscopic evaluations performed during the traditional semen assessment/analysis

Macroscopic evaluations	Microscopic evaluations
Liquefaction	Motility
Appearance	Concentration
Viscosity	Morphology
Volume	Vitality
pH	Non-specific cellular elements

Table 21.2 The lower reference limits (fifth centile and their 95% confidence intervals) for semen parameters

Parameter	Lower reference limit
Semen volume (mL)	1.5 (1.4–1.7)
Total sperm number (10^6/ejaculate)	39 (33–46)
Sperm concentration (10^6/mL)	15 (12–16)
Total motility (PR + NP, %)	40 (38–42)
Progressive motility (PR, %)	32 (31–34)
Vitality (live spermatozoa, %)	58 (55–63)
Sperm morphology (normal forms, %)	4 (3.0–4.0)
pH	≥7.2
Peroxidase-positive leukocytes (10^6/mL)	<1.0

Data from WHO [8]; and Cooper et al [9].

The methods described in this text (Table 21.1) are the ones most commonly used in the andrology setting and is only intended as guidelines. They should not necessarily be taken as obligatory, but it is however important to note that all aspects of semen collection and analysis must be done by properly standardized procedures if the results are to provide valid and useful information. The lower reference limits (fifth percentile and their 95% CI) for the semen parameters measured by these respective analyses are shown in Table 21.2.

Sample Collection

Correct sample collection is important as the results of laboratory measurements depend on it. It is well described that the collection method and the time since the last sexual activity can influence semen quality [13–16]. It is also known that during ejaculation, the first semen fractions voided are more sperm rich as opposed to the later fractions that predominantly consist of vesicular fluid [17].

For the collection of semen for diagnostic or research purposes, it is important to give the man clear instructions

Table 21.3 Summary of the clinical significance of various parameters performed during a routine semen analysis

Routine parameter	Abnormality	Clinical significance (indicative of)	References
Coagulation/liquefaction	Absence of coagulation No liquefaction	Agenesis of the vas deferens Dysfunctional prostate	[1, 11]
Colour/appearance	Yellow Red-brown	Jaundice; drugs Haemospermia	[1, 2]
Volume	High volume Low volume	Inflammation/dysfunction of accessory sex glands Prolonged abstinence period Partial retrograde ejaculation Ejaculatory duct obstruction Short abstinence period	[1, 2, 11]
Viscosity	Hyperviscosity/hypoviscosity	Dysfunctional accessory sex glands	[1, 11]
pH	High pH (>7.8) Low pH (<7.2)	Dysfunctional prostate and seminal vesicles	[3, 8]
Sperm concentration	Low concentration	Short abstinence period Incomplete collection	[3, 4, 8, 9]
Morphology (normal forms)	Teratozoospermia	Abnormal spermatogenesis	[3, 5, 8, 41]
Vitality/viability (live)	Non-viable	Abnormal spermatogenesis Dysfunctional prostate and seminal vesicles	[3, 8]
White blood cells	Leukocytospermia	Male genital tract infection	[1–3, 6, 8]

concerning the collection of the sample. It must be emphasized that the sample needs to be complete, and if loss of any fraction occurs, it must be reported. The sample should be collected after a minimum of 2 days and a maximum of 7 days of sexual abstinence by means of masturbation and ejaculated into a clean wide-mouth container. It is advisable that the sample is collected near the laboratory in order to limit temperature fluctuations and time between collection and analysis. The specimen container must be kept between 20 and 37°C prior to ejaculation and left at either ambient temperature or in an incubator at 37°C while the semen liquefies in order to avoid large changes in temperature that may affect the spermatozoa. It is furthermore important to label the sample thoroughly and record all applicable information in a report. This may include the patient's name, date, period of abstinence, time of collection, interval between collection and start of analysis and completeness of sample amongst others.

Macroscopic Evaluation

The variables that are traditionally assessed during the macroscopic evaluation of a semen sample include liquefaction and appearance, viscosity, volume and pH (Table 21.3) [11]. Inter-patient variability may result in deviations from the normal reference values with a macroscopic assessment. However, ductal obstruction and/or vas/epididymal defects are often the most common reasons for abnormalities of these parameters [11]. Macroscopic analysis should begin preferably at 30 min, but no longer than 60 min after ejaculation.

Liquefaction and Appearance

During sperm collection, the entire ejaculate is collected in one container, where spermatozoa are trapped in coagulation developed from proteins of seminal vesicle origin. This coagulation is subsequently liquefied by the actions of prostatic proteases [17, 18]. Semen naturally liquefies within 60 min if the sample is left at room temperature, and it is normal for semen to begin liquefying after 15 min. The process of liquefaction is significant as it allows for immobilized sperm to become motile [8]. Gelatinous masses may be present in the sample which does not liquefy in the expected period after ejaculation. This phenomenon appears to have no clinical significance [8]. However, it must be recorded in a routine analysis if certain semen samples are not able to liquefy within the required period. Action is then required in order to induce the process of liquefaction through enzymatic digestion or mechanical manipulation of the sample using repeated pipetting [8]. Incomplete liquefaction can be indicative of a disturbance of the accessory sex glands, in particular, the seminal vesicles and prostate. Fibrin, a protein which forms the meshwork of a clot, is responsible for the coagulation of semen directly after ejaculation. Fibrinolysin, a proteolytic enzyme produced by the prostate, acts to degrade the fibrin, which liquefies the congealed semen within 5–15 min. Therefore, if semen fails to liquefy, it can be indicative of a glandular dysfunction of the prostate [19].

The typical appearance of a normal semen sample is a grey-opalescent colour [8]. If a patient has used certain drugs, consumed vitamin supplements or is suffering from jaundice, then the semen may present with a yellow

colour [1]. Semen with a red-brown appearance, known as haemos-permia, is indicative of the presence of erythrocytes in the ejaculate [1, 8].

Viscosity

The viscosity of a semen sample is estimated by aspirating it into a plastic pipette (±1.5 mm diameter) and allowing the semen to drop by gravity and observing the length of any thread formed. A sample is considered normal if it leaves the pipette in small discrete drops. A sample with abnormal viscosity will have drops which form a thread of more than 2 cm long [8]. Viscosity can also be measured by dipping a glass rod into the sample and examining the length of the thread that forms upon removing the rod. If it exceeds 2 cm, it is yet again regarded as abnormal or hyperviscous. Hyperviscosity has been shown to be associated with a lower percentage of motile spermatozoa [20] and is indicative of a deficiency in the functional activity of the accessory sex glands. Changes in the viscosity of semen suggest impairment in both the seminal vesicles and the prostate [1, 21].

Volume

The accessory glands supply their secretions in an ordered sequence to the ejaculate. The total volume of semen is therefore indicative of the functioning of the prostate, seminal vesicles and bulbourethral glands [8, 22]. Deviations from the lower reference value of 1.5 mL (fifth percentile, 95% CI, 1.4–1.7 mL) are therefore symptomatic of a glandular dysfunction [8, 9]. The initial volume of semen is an important parameter in an analysis as the concentration of spermatozoa and other cells in semen is based on the accurate determination of the volume [8]. The volume of a sample can be measured in two ways: firstly, by weighing the sample in the vessel in which it is collected and subtracting the pre-weighed weight of the vessel. This enables to calculate the volume from the sample weight, assuming the density of semen to be 1 g/mL [23, 24]. Secondly, it can be determined by reading the volume from a wide-mouthed graduated cylinder into which it was directly collected [8]. It is advised that the volume should not be determined by decanting the sample into a measuring cylinder or by aspirating it from the container into a pipette or syringe as volume lost can be between 0.3 and 0.9 mL [24, 25].

During the first 4 days following ejaculation, the volume of semen increases at a rate of 11.9%/day [26], and a 2–3 day abstinence period is therefore recommended by the WHO prior to a semen analysis. However, it must not be ruled out that a low volume of semen (hypospermia) can also indicate a pathological condition. A decrease in semen volume is observed in patients presenting with chronic bacterial prostatitis or suffering from retrograde ejaculation [27]. Retrograde ejaculation is referred to as partial or complete flow of ejaculate into the bladder, rather than an antegrade ejaculation through the urethra [28]. This can be due to structural damage to the neck of the bladder or functional impairment of the nerves and neurotransmitters at the bladder neck. Congenital absence of the vas deferens or obstruction of the ejaculatory ducts can also result in a low volume of semen [28]. Seminal fluid volume and thus the semen sample can also decrease with age [29]. Incomplete collection of the semen sample might be another simple explanation for a low semen volume. On the other hand, a high volume of semen, hypervolumeric, can be indicative of an inflammation of the accessory sex glands and is often coupled with poor semen quality [8, 11].

pH

Secretions from the accessory sex glands interact to dictate the pH level of semen. Semen is multi-glandular in origin with the acidic prostatic and alkaline seminal vesicle secretions combining to produce an alkaline fluid with a high pH ranging from approximately 7.2 to 8.0. Hence, abnormal pH levels in a semen analysis can signify glandular dysfunction [30, 31]. The pH is typically assessed with pH paper. In azoospermic patients, a pH value lower than 7.0 can be indicative of bilateral congenital absence of the vas deferens or obstruction of the ejaculatory ducts [8, 32, 33]. A pH with a raised alkaline level of greater than 7.8 can be indicative of dysfunctional seminal vesicles [1].

Microscopic Evaluation

On completion of the macroscopic evaluation, the sample can be microscopically evaluated to determine the number of sperm and non-sperm cellular elements as well as the nature of the spermatozoa (i.e. vitality, motility and morphology—see Table 21.1). A phase contrast microscope is recommended for all examinations of unstained preparations of fresh semen, while a brightfield (×100) oil-immersion objective is required for assessment of morphology.

In order for successful and reproducible microscopic evaluations, it is paramount that representative sampling must occur. This can be achieved by thorough mixing of the sample in the original container by aspirating it ten times into a wide-bore pipette without creating air bubble before aliquots are taken for assessment.

During routine microscopic evaluation, non-specific aggregation of spermatozoa (i.e. immotile sperm to each other or motile sperm to mucus strands, non-sperm cells or debris) can be observed. Agglutination of spermatozoa (i.e. motile spermatozoa sticking to each other) should also be noted as it can be suggestive of anti-sperm antibodies and therefore impinge on motility and concentration assessment [34].

Motility

Motility is an important semen parameter in predicting subfertility as it is indicative of a decrease in the functional competence of spermatozoa [35] and is directly related to the quality of the spermatozoa [1]. Motility can be considered as a physiological variable as it is indicative of the spermatogenesis process [11]. The most recent WHO manual categorizes sperm motility according to a grading system: progressive motility (PR; spermatozoa moving actively, either linearly or in a large circle, regardless of speed), non-progressive motility (NP; all other patterns of motility with an absence of progression) and immotile (IM; no movement) [8]. Motility is typically assessed by a visual determination of a wet-mount slide (20 mm deep) under phase contrast optics at ×200 or ×400 magnification. After the sample has stopped drifting, approximately 200 spermatozoa should be scored [8]. It is also recommended to use an eyepiece reticle with grid to limit the area and allow for the same area of the slide to be assessed. The procedure can be performed at either room temperature or 37°C. CASA is currently also becoming very popular as an objective method of assessing sperm motility. Various factors can affect sperm motility, e.g. oxidative stress, genital infections, accessory sex gland dysfunction and varicocele which can affect motility negatively [1] and can ultimately lead to the impairment of fertility.

The latest lower reference limits for motility (PR + NP) is 40% (fifth percentile, 95% CI 38–42), and for PR is 32% (fifth percentile, 95% CI 31–34) as was determined in fertile men whose partners had a TTP of 12 months or less [9].

Concentration

The concentration or density of a semen sample is the oldest parameter reported to be investigated during a semen analysis [1, 36]. The concentration of semen depends on a variety of factors: the volume of the testes, the period of sexual abstinence prior to ejaculation and the size of epididymal sperm reserve as well as the extent of ductal patency [9]. Sperm concentration is reported in sperm/millilitre (mL) and determined preferably with a 100-mm-deep haemocytometer. The lower limit reference value for the concentration of sperm in a normal semen sample is 15×10^6 mL (fifth percentile, 95% CI 12–16×10^6) while that for the total number of spermatozoa per ejaculate is given at 39×10^6 (fifth percentile, 95% CI 33–46×10^6) [8, 9]. A low concentration as seen in oligozoospermic samples may be indicative of a short period of abstinence as well as incomplete collection of the sample [11]. Certain medical conditions can also impact on the total number of sperm produced, such as diabetes mellitus, cryptorchidism and varicocele [11]. Sperm concentration and sperm number per ejaculate have been shown to be predictors of conception [37, 38] as well as TTP and pregnancy rates [39, 40].

Morphology

A morphological examination provides vital information on the quality of the sperm in a sample. Morphology has been considered to be an essential parameter when establishing the fertility status of a patient [41–43] and can be an important variable in deciding which form of ART will be employed in subfertile patients [11]. During the maturation of spermatozoa, the morphogenetic process can result in imperfections and anomalies which can be seen in a routine semen analysis [42]. It must be considered that several factors can influence the spermatogenesis process such as chemical and environmental factors which could result in anomalies in the morphology of spermatozoa [42]. Therefore, morphologically abnormal spermatozoa in a sample could be indicative of these variables.

Due to the variability in morphology of human spermatozoa, assessment thereof is difficult. Defining the appearance of potentially fertilizing (morphologically normal) spermatozoa was done by examining spermatozoa recovered from post-coital endocervical mucus and the surface of the zona pellucida [41, 44, 45].

The two approaches used to measure sperm morphology to date were the previous WHO guidelines and the Tygerberg strict criteria. The Tygerberg strict criteria were developed on the basis of the morphological assessment of the sperm, especially the acrosomal region [1]. The basis of the criteria is that the morphological appearance, as a result of the size of the acrosome, is a reflection of the physiological and fertilizing capacity of the spermatozoa being graded. A threshold of 14% normal forms was the original reference value for the strict criteria. However, this value has been revised over the past years.

Once a semen smear has air-dried, the slide is stained with the recommended Papanicolaou, Shorr or Diff-Quick staining methods and mounted with a cover slip. For the visualization of the structural features of the spermatozoa, a ×100 oil-immersion brightfield objective and at least a ×10 eyepiece must be used [8]. Currently, the WHO manual classifies the morphology of sperm based on a simple abnormal/normal system as well as the optional test of determining the structural location of the abnormalities. The assessment of the percentage of morphologically normal spermatozoa that are regarded as having fertilizing potential is particularly beneficial in the prediction of a possible pregnancy [8]. The analysis of the parameter that is considered "normal" can therefore be considered to be the spermatozoa with "optimal fertilizing capability" [42]. A threshold of 4% (fifth percentile, 95% CI 3–4) normal forms is the lower reference value as calculated by Cooper [9] and accepted by the WHO [8]. This reference limit is only valid if the classification technique as described in the WHO manual is followed.

By strictly applying certain criteria of sperm morphology, several studies have established relationships between the

percentage of normal forms and various fertility end points such as TTP and successful in vitro as well as natural fertilization rates [41–43, 46–51].

Sperm Vitality

Assessment of vitality, sometimes referred to as viability, indicates the percentage of alive and dead spermatozoa in a sample and is indicative of abnormalities in any of the genital organs [1]. Assessment of the vitality of spermatozoa is recommended if the motility of a sample is less than 10% [11]. Vitality is affected by numerous variables such as abnormal spermatogenesis and vesicular and prostatic fluids [1]. The principle of the test is based on the exclusion of dye by the sperm membrane. In living cells, the membrane remains intact and therefore excludes the dye from penetrating. However, in dead sperm, the membrane's integrity has been compromised, and hence cellular staining by the dye occurs [1]. Sperm vitality should be assessed as soon as possible after liquefaction of the semen sample and within 1 h after ejaculation to minimize environmental effects on vitality. The most common staining method used for vitality testing is the one-step eosin-nigrosin staining technique as described in the WHO manual [8]. The resultant investigation under brightfield optics will show sperm with dark pink heads (dead) and white heads (alive and membrane intact). The hypo-osmotic swelling (HOS) test may be used alternatively to dye exclusion [52]. This method is specifically useful at choosing sperm for intracytoplasmic sperm injection (ICSI) when staining of sperm must be avoided. This method is also based on intactness of cell membranes. Spermatozoa with intact membranes swell within 5 min when placed in a hypo-osmotic medium as indicated by curling of the tails while dead cells show no change. The lower reference limit for vitality is 58% (fifth centile, 95% CI 55–63).

Non-sperm Cellular Elements

Leukocytes are the most commonly observed cells in a semen sample besides spermatozoa [11]. The detection and quantification of leukocytes in semen is not considered part of a routine semen analysis and has little prognostic value in a spermiogram [53]. However, it is useful in a diagnostic sense as it can determine whether uncharacteristic semen parameters are a direct result of the presence of leukocytes [1]. The presence of leukocytes that exceed the WHO reference value of more than 10^6/mL is indicative of a genital tract infection, and the condition is termed leukocytospermia [9]. Certain methodologies for the identification of white blood cells in semen are controversial such as the Papanicolaou and Giemsa stains. This is due to difficulty in discriminating between leukocytes and other cells present in the semen [1]. The peroxidase stain and immunocytochemistry are the two tests that are most commonly employed when testing for leukocytospermia. Red blood cells are also found in a semen analysis, and in high concentrations, they can be indicative of infection and inflammation or possible ductal obstruction [11].

Biochemical Evaluation

The conventional semen analysis is the foundation of determining male factor infertility. However, with the advancement in the understanding of spermatozoa, various in vitro tests have arisen to assess the functional competence of spermatozoa beyond the basic spermiogram [54]. Examples of these tests that are not routinely performed in the traditional semen analysis include sperm–zona interaction, acrosome reaction and the measurement of ROS (reactive oxygen species) production. Their results can be particularly useful in determining the fertilizing ability of spermatozoa beyond the information provided by the basic sperm parameters [54]. These tests are expensive and not commonly performed and are therefore used rather in a research setting than in fertility clinics [11].

Applications to IVF/ART

The results of a semen analysis are the primary step in the assessment of a males' reproductive capacity and is essential in selecting patients for medical intervention such as IVF or ICSI treatment [12, 46]. Therefore, it is essential that the procedures are standardized and carried out precisely to achieve accurate results for the clinician's diagnosis [6].

ICSI has revolutionized the treatment of male infertility as it allows for sperm that would otherwise be incapable of penetrating the ovum to reach the cytoplasm and fertilize the oocyte [46]. This has led to many clinicians undermining the importance of the traditional semen analysis. However, despite the introduction of ICSI in ART treatment, the analysis of semen is still key as it forms the basis of decision-making on the therapeutic options to be taken when dealing with a couple experiencing difficulty in achieving a successful pregnancy [6, 12].

Conclusion

The traditional semen analysis is the established cornerstone of assessing male fertility, and the diagnostic management depends on a sequential, multi-step approach [43]. Recognized reference values for normality are essential due to the relationship between sperm quality and fertility [53]. The information provided by a semen analysis is the least invasive and most cost-effective assessment of a male's fertility status [7]. Despite the introduction of alternative

techniques such as computer-assisted sperm analysis and the advancement of assisted conception, the prediction of fertilization in vitro is still crucial [10].

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Sperm Assessment: Novel Approaches and Their Indicative Value

22

De Yi Liu, Harold Bourne, Claire Garrett, Gary N. Clarke, Shlomi Barak, and H.W. Gordon Baker

Abstract

Standard semen analysis results have limited relationship with results of IVF. We describe alternative tests of sperm and sperm function. Automated semen analysis particularly for sperm morphology, human sperm–oocyte interaction tests and screening tests involving markers of capacitation (hyperactivated motility and protein tyrosine phosphorylation in sperm tails) are potentially useful. Further large studies are necessary before general application.

Keywords

Sperm assessment • Automated semen analysis • Human sperm–oocyte interaction tests • Capacitation • Protein tyrosine phosphorylation

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With the advance of assisted reproductive technology, there have been a number of attempts to develop new methods for the assessment of sperm over those of standard semen analysis (sperm number, motility and morphology), in the hope that they may improve the clinician's ability to manage and predict successful outcomes of treatment. In this chapter, we review a range of these tests, mainly those we have some experience with and discuss other possibilities for the future. The tests discussed can be performed in patients with sperm concentrations above 2×10^6/mL or total sperm count above 5×10^6 per ejaculate. We will not discuss assessment of sperm obtained surgically from the genital tract, patients with severe oligospermia (sperm concentration less than 2×10^6/mL), zero sperm motility, total teratozoospermia where all sperm have the same defect such as an absent heads, sperm autoimmunity and gonadotropin deficiency or suppression.

Before the introduction of ICSI, we conducted a number of studies of the relationship between various sperm tests and the outcomes of standard IVF. Couples with a range of semen abnormalities were included. Excess sperm prepared for insemination were tested and the results examined to determine their association with fertilization rate. Logistic regression analysis was used to show which groups of test results

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were independently significantly related to the fertilization rate. Although most sperm test results had some relationship with the fertilization rate, many were closely correlated, and when entered into the regression model, only a few remained significant. This approach allowed us to determine which sperm characteristics were most important for fertilization. There was a strong relationship between fertilization rate and sperm morphology assessed with the strict approach and some other factors such as DNA normality assessed with acridine orange florescence, automated sperm motility particularly straight line velocity (VSL) and the ability of sperm to bind to and penetrate the zona pellucida (studies summarized in [1, 2]).

The sperm–zona pellucida interaction results were most significantly associated with fertilization rates, and this led us to develop tests of sperm function using human oocytes which had failed to fertilize or were unsuitable for insemination or injection. Results of these tests confirmed that defective sperm–zona pellucida interaction is a major cause of failure of fertilization with standard IVF [3–8]. We provide details of the methods as any group associated with a clinical IVF programme could perform these tests.

Sperm Motility and Morphology

Several tests related to standard semen analysis measures of motility and morphology have been developed, but as yet none has an established place in patient management.

Hypoosmotic Swelling Test

Some investigators promoted hypoosmotic swelling as a predictor of IVF results, but we found results of this test were not significant when other sperm measures were taken into account [1].

DNA Normality

DNA or chromatin normality can be assessed by a variety of techniques including TUNEL and comet assays, but simpler methods involving sperm head decondensation and staining with dyes produce correlated results. We found little relationship between sperm head decondensation or aniline blue staining and fertilization rates, but acridine orange fluorescence assessed by microscopy was significantly related independent of sperm morphology and sperm–zona pellucida binding [1]. Subsequently, we investigated the use of the flow cytometry method involving acid denaturation of sperm and found that the increase in sperm with red fluorescence (the DNA fragmentation index, DFI) was inversely correlated with fertilization rates with standard IVF, but not with those of intracytoplasmic sperm injection. DFI was correlated with sperm motility, viability and morphology [9]. We showed the zona pellucida selectively binds sperm with normal acridine orange staining [10]. Meta-analysis of the results of the DFI test indicates that it is not a great advance over standard semen analysis. Also, claims that DFI results predict poor outcomes with implantation and subsequent pregnancy loss are unconvincing [11].

Sperm Movement Characteristics

We found several motility measurements assessed by computer-assisted semen analysis (CASA) were related to fertilization rates with standard IVF, particularly VSL [12]. Also, VSL and automated sperm morphology %Z (see below) together were the only independently significant semen characteristics related to natural conception rates in subfertile couples [13]. We believe there is potential for VSL to be included as a clinically useful semen analysis characteristic, but further large studies are required for confirmation.

Automated Sperm Morphology

Early studies showed that among the standard semen variables, sperm morphology was the strongest predictor of fertilization rates with IVF. We found that morphology was critical for sperm binding to the zona pellucida and that the population of sperm bound to the zona pellucida had higher normal morphology than those in the insemination medium [14–17]. We developed an automated method for computer image analysis of sperm morphometry and used the characteristics of sperm that most differentiated those bound to the zona pellucida from those in the insemination medium to develop a measure of sperm with zona binding capacity (%Z) as an overall assessment of sperm morphometry [13, 18]. Sperm morphometry should greatly improve the predictive value of semen analysis. Currently sperm morphology is assessed subjectively, and there are extreme variations between laboratories that are obvious in external quality assurance results. This should be improved by automation, but all current commercial CASA systems with sperm morphology modules may not give results comparable with those of manual methods or provide a measure equivalent to %Z.

Sperm Function Tests

Developing sperm function tests that would accurately predict fertility and the results of IVF has been an active interest of several groups for some time, but no test has become widely used because it either failed to live up to its promise

or the assay is difficult, largely due to limited supply of human material for the tests and the unavailability of substitutes. For example, recombinant human zona proteins have been found inactive or inconsistently active [19].

Hamster Oocyte Penetration Test

Acrosome-reacted human sperm fuse with the oolemma of zona-free hamster oocytes and undergo nuclear decondensation in the ooplasm. This test is useful for research purposes [20]. Although promoted early as a human sperm function test that would predict the ability of sperm to fertilize in vitro, this was found not to be the case [21]. As discussed below with sperm defects, the main mechanism of interference with fertilization is reduced ability of sperm to bind to the zona pellucida or undergo the acrosome reaction on the zona pellucida. Defects of sperm–oolemma binding are uncommon [2]. Searches for oocytes from other species that could be used for testing human sperm binding and penetration or genetic modification of animal zona proteins that would allow human sperm interaction have been unsuccessful.

Human Sperm–Oocyte Interaction

We developed methods for testing stages of human fertilization using human oocytes that failed to fertilize in clinical IVF (Fig. 22.1). These tests improve evaluation of sperm function and have resulted in our identification of two defects of sperm–zona pellucida interaction: (1) low ability of the sperm to bind to the oocyte, called defective sperm–zona pellucida binding (DSZPB), and (2) failure of the acrosome reaction, called disordered zona pellucida-induced acrosome reaction (DZPIAR). Our reviews provide summaries of the results of applying these tests to study the signal transduction and effector pathways of the acrosome reaction and also describe extensions such as using different fluorescent dyes to stain test and control sperm [1, 2]. The method we currently use can measure both sperm–zona pellucida binding and the zona pellucida-induced acrosome reaction in the same test (Fig. 22.2). Oocytes that have failed to fertilize with standard IVF and immature oocytes (GV or MI) unsuitable for ICSI are used. The zona pellucida of these oocytes has similar biological activity as those of normal mature oocytes to bind sperm and induce the acrosome reaction [2]. The oocytes are used fresh or after storage in 1 M ammonium sulphate. Salt-stored oocytes are washed and incubated for 2 h with 2×10^6 motile sperm in 1 mL human tubal fluid medium with groups of four oocytes. Unbound sperm are removed by washing oocytes with a wide-bore pipette and sperm tightly bound to the surface of the zona pellucida are counted. Where there is normal sperm–zona pellucida binding, sperm bound to the surface of each zona pellucida are then removed by repeated aspiration of the oocyte with a narrow-bore pipette (120 μm, slightly smaller than the oocyte) with a small volume of medium placed on a slide and stained with fluorescein-labelled *Pisum sativum* agglutinin (Fig. 22.2). Two hundred sperm are counted for each test. Sperm penetrating the zona pellucida are counted, and the motility of sperm in the medium and the spontaneous acrosome reaction are also assessed at the end of the incubation period. The technician performs the counts blind to the condition of the patient. Assay reproducibility has been studied for the same sperm samples exposed to oocytes obtained from different IVF patients and for repeated ejaculates on different days from the same DZPIAR patients and normal men. For both, the variation in results is low (SD <10% [8]). Fresh and salt-stored oocytes give the same results, as do oocytes with or without sperm penetrating the zona pellucida from previous IVF. Under the conditions of the test, fertile men have >100 sperm tightly bound to each zona pellucida, and an average of ≤40 bound sperm/zona pellucida is defined as low. Using human tubal fluid medium plus 7–10% human serum, an average of 48% of zona pellucida-bound sperm undergoes the acrosome reaction, and a result of <16% is defined as low. Different culture media may have different effects on the acrosome reaction, and thus, the normal reference values depend on the medium and protein supplement. Because we use oocytes discarded from IVF, we have performed extensive studies on the effect of oocyte variability on the results of these tests [2]. We use four oocytes for each sperm sample because occasional oocytes will not bind

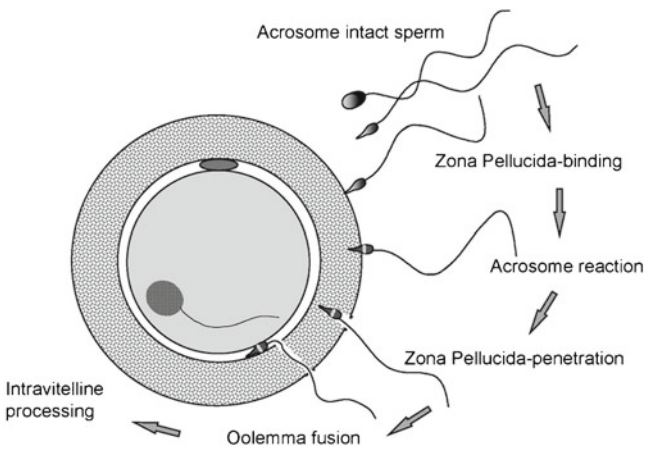

Fig. 22.1 Diagram of human sperm–oocyte interaction. Motile sperm swim through medium and cumulus oophorus (not shown) to attach to the surface of the zona pellucida. The acrosome reaction is stimulated, and the plasma and outer acrosomal membranes are shed as the sperm penetrates the zona pellucida. In the perivitelline space, the sperm plasma membrane persisting over the equatorial segment fuses with the oolemma, then the sperm is engulfed into the vitellus, and the sperm head decondenses

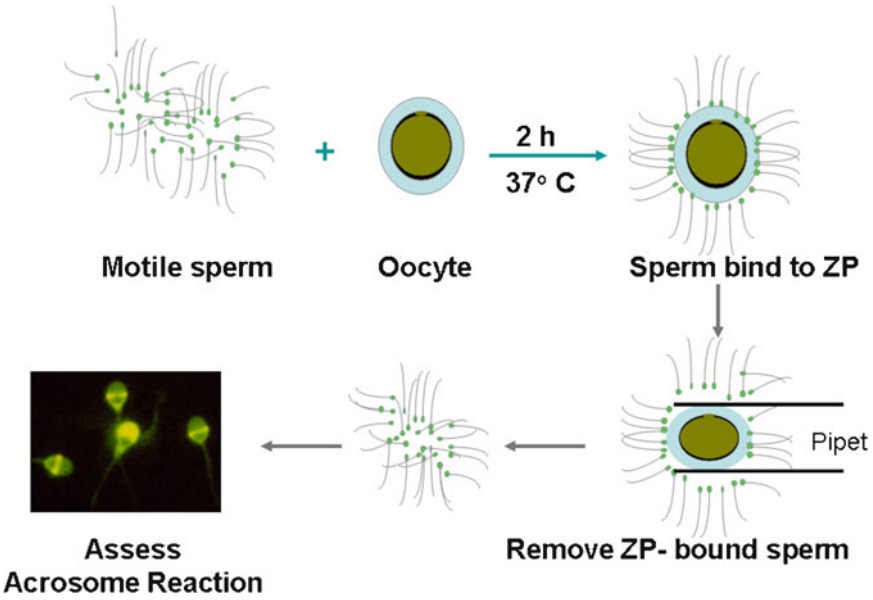

Fig. 22.2 Flow diagram of human sperm–oocyte interaction tests. Motile sperm are incubated with oocytes and sperm tightly bound to the zona pellucida counted after washing the oocytes to dislodge loosely adherent sperm. Sperm bound to the zona pellucida are removed by pipetting the oocyte with a pipette of diameter slightly smaller than that of the oocyte. Sperm penetrating the zona pellucida remain and are counted. The dislodged zona-bound sperm can be further assessed, for example, with fluorescein-labelled *Pisum sativum* agglutinin for acrosome status. Illustrated are one sperm with intact acrosome showing uniform florescence of the anterior half to two-thirds of the head and three acrosome-reacted sperm showing a florescent band in the equatorial region

sperm. We confirm abnormal results by repeating the test on another semen sample.

Defective Sperm–Zona Pellucida Binding

Low sperm–zona pellucida binding can be classified into two types according to semen analysis results: DSZPB types I and II [2, 8]. Type 1 has abnormal semen analysis: oligozoospermia, asthenozoospermia, teratozoospermia or a combination. About 80% of patients with DSZPB type I have defects of sperm head shape that are obvious in routine semen analysis and presumably account for the low sperm–zona pellucida binding. In contrast, DSZPB type II has normal semen analysis, and the diagnosis can only be made by testing sperm–zona pellucida binding using human oocytes. About 13% of infertile men with normal semen analysis results have DSZPB type II [2, 4, 8]. It is likely that DSZPB originates from abnormal spermatogenesis or sperm maturation.

Disordered Zona Pellucida-Induced Acrosome Reaction

We found a group of infertile men with consistently normal routine semen analysis and normal sperm–zona pellucida binding, but sperm did not penetrate the zona pellucida because of a low acrosome reaction on the zona pellucida. We first identified this condition in a group of patients with unexplained infertility and repeated zero or low fertilization rates (<30%) with standard IVF [6, 22]. Normally an average of 48% (range 20–98%) of sperm bound to the zona pellucida undergo the acrosome reaction during the 2-h incubation involved in the test [6]. In contrast, with DZPIAR, very low proportions (mean 6%, range 1–16%) of sperm undergo the acrosome reaction on the zona pellucida. Studies on the frequency of DZPIAR in patients with unexplained infertility indicate about 25% are affected [6, 22]. Interestingly we have found that the zona pellucida-induced acrosome reaction is correlated with sperm concentration in the ejaculate, and although the patients had by definition sperm concentrations greater than 20×10^6/mL, those between 20 and 60×10^6/mL had significantly lower zona pellucida-induced acrosome reaction. Low zona pellucida-induced acrosome reaction is also frequent in oligospermic (<20×10^6/mL) and severely teratospermic (normal sperm morphology <5%) men with normal sperm–zona pellucida binding [5, 7].

Human Sperm–Oolemma Binding

It is possible to remove the zona pellucida from the oocyte with dilute acid treatment and then use a similar approach to sperm–zona pellucida binding (Fig. 22.2) to study sperm binding to the oolemma. We found evidence that sperm needed to be acrosome-reacted before binding to the oolemma and that globospermic sperm would not bind. However, we found no other patients with a specific defect

of sperm–oolemma binding, making this test of little clinical value [1, 23].

Tests Correlated with Sperm Function

As well as the strong relationship between normal morphology and sperm–zona pellucida binding, we have found that two markers of sperm capacitation, hyperactivated motility and protein tyrosine phosphorylation in sperm tails, are correlated with the zona pellucida-induced acrosome reaction and sperm–zona pellucida binding.

Hyperactivation and Sperm–Zona Pellucida-Induced Acrosome Reaction

The proportion of sperm which undergo hyperactivation in culture medium assessed by CASA (Hamilton Thorne sperm analyser) is highly correlated with the zona pellucida-induced acrosome reaction, but not with sperm–zona pellucida binding capacity [24].

Protein Tyrosine Phosphorylation and Sperm–Zona Pellucida Binding

Tyrosine phosphorylated proteins increase mainly in the principle piece of human sperm with capacitation. There is a significant correlation between human sperm–zona pellucida binding and tyrosine phosphorylation in human sperm detected with the monoclonal antibody PY20 labelled with fluorescein [25].

Thus, these simple tests of hyperactivation and protein tyrosine phosphorylation, which could be performed as an addition to routine semen analysis, may be useful to screen for defects of human sperm–zona pellucida interaction. However, large prospective studies are needed to confirm this.

Summary and Conclusions

A number of sperm tests of varying complexity have been reviewed. Standard semen analysis remains the most useful clinically but has limited relationship with results of IVF. Sperm morphology assessed by the strict method is valuable for predicting poor fertilization with standard IVF, but its subjectivity causes problems with some laboratories failing to find it useful. Objective methods involving automated computer image analysis are being developed. Methods for assessing human sperm–oocyte interaction have been described, and these can be used for diagnosis. Some methods such as hamster oocyte penetration tests have been discarded, and others such as sperm DNA testing are of uncertain value.

While none of the tests discussed here have become established as useful in clinical practice, we believe automated semen analysis particularly for morphometry has great potential. We use the human sperm–oocyte interaction tests in patients with idiopathic infertility to detect those who have DSZPB or disordered sperm–zona pellucida-induced acrosome reaction and direct these patients to ICSI to avoid IVF treatments with low or zero fertilization rates. We also use these tests for assessing patients with isolated marginal sperm morphology. Those with confirmed poor zona binding require ICSI, while those with normal sperm–oocyte interaction can be treated by standard IVF. Further studies are necessary to determine if hyperactivated motility and immunofluorescence staining of tyrosine phosphorylation in sperm tails would be useful screening tests for the defects of human sperm–oocyte interaction.

Because of the range of abnormalities of human sperm and the variability in test results from day to day within the same patient, it is unlikely that a single predictive test that can be applied once will be found. Clinical information about the couple, together with a panel of repeated sperm test results, will always be required for predicting male fertility.

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Sperm Processing for IVF

23

Ralf Henkel

Abstract

The first sperm separation methods that were developed only comprised of one or two washing procedures to eliminate seminal plasma with subsequent resuspension of the male germ cells. Following these first reports on human sperm processing, more sophisticated methods were developed in order to obtain sufficient amounts of motile, functionally competent spermatozoa for IVF, one of them being a swim-up procedure from a washed cell pellet. Except for the first washing procedures, modern sperm processing techniques can be differentiated in migration, filtration, and density-gradient centrifugation. While for all migration techniques (conventional swim-up and migration-sedimentation), the sperm cells' most obvious feature, self-propelled movement, is an essential prerequisite; the separation principle in the filtration and density-gradient centrifugation techniques is based on a combination of the sperms' own motility and their adherence to filtration matrices and the retention at phase borders, respectively. Apart from possible financial considerations, sperm physiology and the physiology of the fertilization process have to be taken into account for any method of assisted reproduction in order to better select functional sperm or better protect and "preserve" sperm functions from damages caused by the separation process.

Keywords

Sperm processing in IVF • Sperm separation methods • Swim-up procedure in IVF • Migration-sedimentation technique in IVF • Density-gradient centrifugation for sperm

Introduction

The birth of Louise Brown, the first human who was born on 25 July 1978 after the application of assisted reproduction techniques hallmarks the advent of the era of assisted reproductive technology (ART) in the human [1]. The first in vitro fertilization (IVF) cases, including that of Louise Brown, were performed to treat female tubal infertility. Subsequently, the demand for assisted reproduction techniques grew dramatically with the increasing number of men showing poor semen quality. Hence, scientists and clinicians were prompted to develop a wide array of different laboratory techniques focusing on the selection and enrichment of motile and functionally competent spermatozoa from the ejaculate. Since our understanding of the fertilization process and various sperm functions also extended significantly and the percentage of andrological cases with even poorer semen quality increased rapidly, the initial simple sperm separation techniques were not sufficient enough anymore, and new improved sperm separation techniques had to be developed.

Spermatozoa are not only the smallest but also the most polarized cell in the body (sperm head and a flagellum) that even fulfill their physiological functions, fertilization of oocytes, outside the body in a different individual, the female

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genital tract. Thus, the male germ cell is a very specialized and in certain aspects very sensitive cell. The latter is based on the sperm cell's special composition of its plasma membrane with an extraordinary high amount of poly-unsaturated fatty acids, particularly docosahexanoic acid with six double bonds in the molecule [2]. This feature is the foundation of the high membrane fluidity which is essential in maintaining the extreme polarization of the sperm and is required for normal sperm function [3]. Together with the sperm cell's lack of intrinsic reactive oxygen species (ROS)-scavenging systems due to a lack of cytoplasm, which is harboring repair mechanisms in any other cell, this renders the male germ cell extremely susceptible to oxidative stress by ROS [4] impairing the membrane function and resulting in loss of fertilizing potential [5, 6], serious damage of the DNA [7, 8] or even cell death.

In vivo, after normal ejaculation for sexual intercourse, spermatozoa depend on scavenging systems provided by the seminal plasma, which is the biological fluid that contains more antioxidant substances than any other physiological fluid does. The most important natural antioxidants in seminal plasma seem to be vitamin C and E [9, 10], superoxide dismutase [11], uric acid [12], glutathione [13], or the polyamine spermine that acts directly as a free radical scavenger [14]. If these protective substances are removed by means of any sperm separation technique, this can cause severe damage to the sperm cell and its membranes since sperm functions like motility or acrosome reaction are basically membrane functions. This damage is set by ROS either by the sperm cells themselves or leukocytes [15–17], which produce about 1,000 times more ROS than spermatozoa [18, 19].

On the other hand, even the extreme polarization of the male germ cell can be a direct target of damage through excessive centrifugation, resuspension, and vortexing of semen samples [15, 16, 20] as the mechanical stress imposed on the sperm can be harmful to the cell and its functions leading not only to decreased percentages of motile and vital sperm but also to a reduced mitochondrial membrane potential, which is essential for normal sperm function [21–23].

Sperm Processing Methods

In vivo, separation of motile sperm most capable of fertilizing oocytes from immotile sperm, debris, seminal plasma, and leukocytes is taking place in the female genital tract by active migration through the cervical mucus [24]. Yet, this process does not only separate motile and potentially fertile sperm, but it also prepares and enables male germ cells for the fertilization process by means of fundamental physiological changes called capacitation. In turn, the capacitation process involves, among others, changes in the motility pattern, metabolism, and the removal of cholesterol from the plasma membrane leading changed fluidity of the sperm plasma membrane, eventually enabling the sperm to undergo acrosome reaction and thus penetrate and fertilize oocytes [25–29].

The first sperm separation methods that were developed only comprised of one or two washing procedures to eliminate seminal plasma with subsequent resuspension of the male germ cells [30]. Following these first reports on human sperm processing, more sophisticated methods were developed in order to obtain sufficient amounts of motile, functionally competent spermatozoa for IVF, one of them being a swim-up procedure from a washed cell pellet.

Except for the first washing procedures, modern sperm processing techniques can be differentiated in migration, filtration, and density-gradient centrifugation. While for all migration techniques (conventional swim-up and migration-sedimentation), the sperm cells' most obvious feature, self-propelled movement, is an essential prerequisite; the separation principle in the filtration and density-gradient centrifugation techniques is based on a combination of the sperms' own motility and their adherence to filtration matrices and the retention at phase borders, respectively.

Apart from possible financial considerations, sperm physiology and the physiology of the fertilization process have to be taken into account for any method of assisted reproduction in order to better select functional sperm or better protect and "preserve" sperm functions from damages caused by the separation process.

Criteria for a "good" sperm selection are as follows:

- Elimination of seminal plasma, decapacitation factors, and debris
- Elimination/reduction of dysfunctional and ROS-producing sperm
- Elimination/reduction of leukocytes
- Elimination/reduction of bacteria
- Enrichment of functional sperm in terms of motility, DNA integrity, acrosome reaction, and normal sperm morphology
- Cost-effectiveness
- Easy and quick to perform
- Allow processing of larger volumes of ejaculates

However, since none of the different methods available meets all the criteria, it is mandatory for every Andrology laboratory/IVF unit to be able to perform a variety of different sperm separation methods having the individual circumstances of the patients as first priority in mind.

Liquefaction of Ejaculates and Viscous Ejaculates

An essential requirement for any sperm preparation method to be performed is that the semen is liquid. Normally, human semen liquefies after 15–30 min, and these ejaculates can

directly be subjected to a sperm processing technique. In the laboratory, semen liquefaction is normally performed by placing the semen sample for 30 min into an incubator at 37°C. However, if the ejaculate does not liquefy after 60 min, which can be the case in about 10% of infertile patients [31], seminal viscosity remains high and can severely interfere with sperm motility resulting in poor yield of motile sperm after sperm separation.

Semen viscosity, which is sometimes referred to as "consistency," can be tested by means of gently aspirating a semen sample into a Pasteur pipette and then allowing the ejaculate dropping out. Normally, the seminal fluid is running out in discrete drops. Threads longer than about 2 cm are indicative of high viscosity and poor liquefaction, i.e., viscosipathy [32]. Since such ejaculates cannot well be processed, liquefaction can be achieved by enzymatic digestion using bromelain (1 mg/mL) [32] or a small spatula tip of approx. 2–5 mg crystalline α(alpha)-chymotrypsin to be mixed into the semen and incubated for 15–30 min at room temperature [33]. However, one still has to consider that these enzymes might be harmful to the sperm or oocyte and have to be removed by washing procedures as soon as possible. Alternatively, viscous semen can also be mixed with IVF medium in order to decrease its viscosity.

NB: Recommendations to force viscous seminal fluid through 18 or 23 gauge needles should be refrained from since such procedure can severely damage sperm.

Swim-Up

Since the introduction of the so-called conventional swim-up procedure by Mahadevan and Baker [34], this method has been successfully used in many IVF units around the world with excellent fertilization rates and is still in use for all techniques in assisted reproduction including intrauterine insemination (IUI) and IVF. This method is simple and cheap and does not require sophisticated equipment or highly specialized skills.

Procedure

Typically, 1 mL of the liquefied semen is mixed with 4 mL of IVF medium containing 10 mg/mL serum albumin or 10% inactivated serum and centrifuged for 10 min at 300×*g*. Then, the supernatant is carefully taken off with a pipette and discarded. For this step, it is essential that the surface of the pellet is not disturbed or that parts of the pellet are sucked into the pipette as this would result in a decreased yield and motility in the sperm suspension. Subsequently, fresh medium is layered very carefully by letting 0.5–1 mL slowly rinse down the inner surface of the test tube tilted at an angle of about 45° on top of the pellet and incubated for 30–60 min at 37°C in an incubator. After this incubation period, the test tube is taken out of the incubator, and the medium is carefully aspirated into a pipette again without disturbing the pellet which can become quite soft during the incubation period. Ideally, sperm fractions with >90% motility can be obtained using this technique.

NB: Considering that the success of the swim-up technique strongly depends on the initial motility in the ejaculate and the size and quality of the surface of the pellet obtained after the centrifugation step, the yield of this technique is rather limited. Therefore, it is advisable to run two or three or more tubes from one semen sample at the same time in order to increase the yield. Since the use of medium that does not contain macromolecules like albumin is detrimental to spermatozoa, it is not advisable to use unsupplemented medium as it might result in sperm sticking to the surface of the test tube leading to a lower yield and motility.

Advantages/Disadvantages of the Technique

Normally, this sperm selection method recovers a very clean fraction of highly motile sperm that can directly be used for insemination in IVF or for IUI. For the latter, it is important that not more than 0.5 mL of the isolated sperm fraction after swim-up is inseminated into the uterus as higher volumes might leak out.

With regard to the quality of the ejaculates, the conventional swim-up technique is rather restricted to good quality ejaculates with high sperm concentration and good motility. With regard to the application of the swim-up for artificial insemination, it is not only important to know that the yield is rather low but also that sperm can be massively damaged by ROS because of the close cell-to-cell contact during the centrifugation step. This is particularly the case in patients with male genital tract infections. If, despite good motility and sperm count in the ejaculate, the expected sperm concentration is less than 10^6/mL after the swim-up, one should consider the effect of ROS.

Migration-Sedimentation

The migration-sedimentation technique, which was originally developed by Tea et al. [35], is a more sophisticated method of sperm processing. Like the swim-up technique, it is based on the sperm cells' own motility, yet combined with a sedimentation step. However, special, commercially available "Tea-Jondet tubes" have to be used. Contrary to the conventional swim-up method, sperm swim up directly from the liquefied semen sample into the supernatant medium, deposit, and accumulate into the inner cone of the tube. Since such a

migration technique directly from the semen is the gentlest method of sperm processing, several modifications have been developed, which are even used for ICSI [36–38].

Procedure

In order to process semen for IVF using the migration-sedimentation technique, the "Tea-Jondet tubes" are filled up with 1–2 mL IVF medium supplemented with 10 mg/mL serum albumin or 10% inactivated serum. The overlaying medium must not be too high above the edge of the inner cone. Then, approx. 0.5 mL of liquefied semen is placed in the outer ring around the cone carefully paying attention that no ejaculate is running into the inner cone. After incubating the tubes for 1–2 h at 37°C, the supernatant medium, including the medium in the inner cone, is carefully aspirated with a Pasteur pipette without aspirating remnants of the ejaculate. Typically, sperm fractions with motility rates of more than 90% can be obtained and after adjustment of sperm concentration directly used for insemination.

For the use in ICSI, the whole liquefied ejaculate (or part of it) of the respective patients is centrifuged for 10–15 min at 300×*g*, and the supernatant except for about 0.3–0.5 mL which contains the sperm is discarded. This "concentrated" ejaculate is filled into the "Tea-Jondet tubes" as described above and incubated. In order to obtain higher numbers of motile sperm, processing can be carried out in duplicate.

NB: Like in the swim-up method, the success of isolating motile sperm using migration-sedimentation is dependent on initial quality of the ejaculate. For its application in ICSI, it is, however, important that the semen must *not* be diluted with medium for centrifugation. The seminal plasma is then still able to have at least some protection against oxidative stress.

Advantages/Disadvantages of the Technique

Migration-sedimentation is one of the gentlest methods of processing sperm and usually results in a very clean fraction of highly motile, functional sperm.

Considering the very low recovery rate in its original protocol, this method is rather restricted to higher quality ejaculates. The technique is also a bit more expensive than the conventional swim-up and requires advanced skills.

Glass Wool Filtration

Using a different approach Paulson and Polakoski [39] succeeded to separate motile from immotile sperm by means of densely packed glass wool fibers. The principle for the separation of motile spermatozoa from immotile, debris and leukocytes is not only rested in the self-propelled movement of the sperm cells but also on a filtration effect of the glass wool fibers, for which both are responsible, a mechanical retention of the bigger particles in the ejaculate as well as the adhesion of these particles to the surface of the glass fibers [40]. Hence, the kind of glass wool used plays a cardinal role in the success of this technique, and it is not possible to use any kind of glass wool for sperm processing [41]. This adhesion and filtration process is a feature of glass wool filtration that may contribute to the selection of spermatozoa with matured nuclei, i.e., with good chromatin condensation [42].

Recently, Grunewald et al. [43] developed a glass wool, which chemically activated to bind annexin V to the surface of the glass wool fibers. Annexin V binds to externalized phosphatidyl serine and is an indicator of apoptotic cells [44, 45]. While the conventional glass wool filtration is in routine use in many IVF centers, clinical data supporting the efficiency of this new molecularly activated glass wool remains to be seen.

Procedure

Useful glass wool is available from Manville Fiber Glass Corp. (code 112; Denver, CO) or glass wool columns from TransMIT (SpermFertil®; Giessen, Germany). While from the former, 15–30 mg of glass wool has to be loosely packed in a Pasteur pipette or small syringe, the latter are sterile, ready-to-use glass wool columns. In order to eliminate loose glass particles, both columns have to be flushed carefully with at least 2 mL sterile IVF medium. Subsequently, the glass wool column must be put on a new sterile test tube and filled with 1–3 mL *liquefied* fresh semen. Filtration should take place in an incubator at 37°C with occasional visual control in order to avoid the column falling dry. Immediately after the semen has passed through the glass wool, the column is washed with 0.5 mL fresh IVF medium. Finally, the filtrated semen will be diluted in the ratio 1:5 with fresh IVF medium and centrifuged for 10 min at 300×*g*. The supernatant will then have to be carefully discarded and the pellet resuspended in fresh IVF medium. For better washing of sperm, this procedure can be repeated. This sperm suspension can then be used for insemination after adjusting sperm concentration.

NB: As high concentrations of "particles" might block the column and prevent semen from being filtrated properly, the amount of semen used depends on sperm and debris concentration. Therefore, in cases of high "particle" concentrations, the ejaculate can be divided on 2–3 glass wool columns for filtration and the filtrates eventually be combined. For gentle sperm processing using glass wool filtration, it is fundamentally important that the sperm separation takes place first. Only afterward, washing of sperm should take place.

Advantages/Disadvantages of the Technique

Principally, glass wool filtration is an easy to perform technique, which results in the recovery of spermatozoa with good motility. Since the whole ejaculate can be filtrated, ejaculates from patients with oligozoospermia can be processed. In addition, the technique eliminates up to 90% of leukocytes contaminating the semen and therefore reduces ROS significantly [46]. As a disadvantage, it can be brought bear that the filtration does not result in as clean fractions as the swim-up procedure does. There is always some debris or immotile sperm that pass through the mesh.

Density-Gradient Centrifugation with Different Media

Density-gradient centrifugation is another method for gentle sperm separation. Its principle is based on the ability of motile sperm cells to penetrate concentration boundaries in direction of the centrifugation force. This penetration is quicker the faster sperm swim; thus, highly progressively swimming sperm are reaching the bottom of a centrifuging test tube quicker than immotile or poorly motile sperm, which are retained at the boundaries of interphases.

The first reports employing density-gradient centrifugation to isolate progressively motile human spermatozoa date back to 1981 and 1983 [47, 48]. Initially, Percoll®, a medium containing polyvinylpyrrolidone-coated silica particles, was used to create media with different densities. Since 1996, however, Percoll® has been withdrawn from the market for use in assisted reproduction because of its possible risk of being contaminated with endotoxins [49, 50]. Yet, other density media have emerged from the market with good results in separating highly motile sperm, competent for fertilization. These new density media are based on silane-coated silica particles and have been proven to have very low toxicity. The most commonly used new density media are SilSelect® (FertiPro N.V., Beernem, Belgium), SupraSperm® (MediCult, Jyllinge, Denmark), PureSperm® (NidaCon International AB, Mölndal, Sweden), or ISolate® (Irvine Scientific, Santa Ana, CA, USA).

Like for glass wool filtration, the selection of nuclear matured sperm in terms of chromatin condensation has been described for density-gradient centrifugation [51, 52]. Moreover, while recent studies showed that processing of sperm with PureSperm® resulted in significantly lower percentages of sperm exhibiting DNA damage and extended survival rate [53, 54], older studies using Percoll® rather revealed adverse effects on the sperm DNA [55, 56]. Moreover, for the high motility fraction of density gradient prepared spermatozoa lower percentages of sperm with externalized phosphatidylserine and disrupted mitochondrial membrane potential have been described [57].

Procedure

There are two different kinds of density gradients that can be performed, the continuous or the discontinuous. Nowadays, most labs are using the discontinuous gradient employing a two-step gradient consisting of 40% (45%) and 80% (90%) density medium. These density gradients can either be bought ready to use or can be made from high-concentration stock solutions. Depending on the product used, the two-step gradient is prepared by *carefully* overlaying 1–2 mL of the high-density solution (80%/90%) with 1–2 mL of the low-density solution (40%/45%). A distinct phase border between the two layers must be visible. After the gradient is equilibrated in 5% CO_2 at 37°C in an incubator, 1 mL of the liquefied and thoroughly mixed semen sample is *carefully* dispensed on top of the prepared gradient. Again, a distinct phase border should be visible. Subsequently, the gradient is centrifuged for 20–30 min at 300–400×*g*.

After this centrifugation step, the soft pellet can be obtained in two ways. Firstly, by carefully aspirating the pellet with a Pasteur pipette. By applying this procedure, nothing of the overlaying layers should be aspirated. Secondly, the top layers of the gradient can be removed and discarded first, and then the pellet can be aspirated and placed into a clean test tube. This sample has then to be resuspended in 3–5 mL fresh sperm preparation or IVF medium and centrifuged 10 min at 300–400×*g* again. This procedure has to be repeated twice. After adjusting sperm concentration, the finally resulting sperm suspension can be used for insemination.

NB: Overloading the gradient with either too much volume or a too high number of "particles" (spermatozoa and/or debris) will result in poor sperm separation. On the other hand, in cases of oligozoospermia or asthenozoospermia, more than one test tube can be prepared in order to obtain a sufficient number of competent sperm for fertilization.

Advantages/Disadvantages of the Technique

Normally, density gradient centrifugation results in a good and clean fraction of highly motile spermatozoa with good recovery. Even ejaculates with low sperm counts from patients with oligozoospermia can be processed. The possibility to process the full volume of the ejaculate increases the yield. Considering that the technique eliminates leukocytes to a large extent, ROS are significantly reduced.

The formation of good interphases between the different media gradients is essential for the quality of the sperm separation and needs special attention during the preparation. After the density-gradient centrifugation the "soft pellet" containing the high-quality sperm has to be washed in order to eliminate the density medium. Thus, an additional centrifugation step is required.

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PESA/TESA/TESE Sperm Processing

24

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Abstract

Spermatozoa can be retrieved from either the epididymis or the testis, depending on the type of azoospermia, using different surgical methods such as PESA, TESA, TESE, and micro-TESE. After collecting the epididymal fluid or testicular tissue, laboratory techniques are used to remove contaminants, cellular debris, noxious microorganisms, and red blood cells. Processed spermatozoa may be used for intracytoplasmic sperm injection or eventually be cryopreserved. However, spermatozoa collected from either the epididymis or the testis are often compromised and more fragile than ejaculated ones. Therefore, sperm processing techniques should be used with great caution to avoid jeopardizing the sperm fertilizing potential in treatment cycles. In this chapter, we describe methods for the optimal processing of surgically retrieved specimens, either fresh or frozen–thawed. We also provide tools to aid in the identification of viable immotile spermatozoa for ICSI. The clinical outcomes of ICSI using testicular and epididymal sperm in azoospermic men are presented, and the tips and pitfalls of sperm processing techniques for surgically retrieved specimens are critically discussed.

Keywords

PESA/TESA/TESE sperm processing • Sperm processing with PESA/TESA/TESE • ICSI spermatozoa identification • Epididymal sperm aspiration • Sperm processing techniques • Intracytoplasmic sperm injection • Azoospermia • Epididymal spermatozoa • Testicular spermatozoa

Two major breakthroughs occurred in the area of male infertility only 2–3 years apart [1–3]. The first was the development of intracytoplasmic sperm injection (ICSI) for the treatment of male factor infertility due to severely abnormal semen quality [1]. The second was the extension of ICSI to azoospermic males and the demonstration that spermatozoa derived from either the epididymis or the testis were capable of normal fertilization and pregnancy [2, 3]. In the case of azoospermia, two totally different clinical situations exist, i.e., obstructive and nonobstructive azoospermia (NOA). In obstructive azoospermia (OA), spermatogenesis is normal, but a mechanical blockage exists in the genital tract, somewhere between the epididymis and the ejaculatory duct, or the epididymis and vas deferens are totally or partially absent. Causes of OA are acquired or congenital. Acquired OA may be due to vasectomy; failure of vasectomy reversal; postinfectious diseases; surgical procedures in the scrotal, inguinal, pelvic, or abdominal regions; and trauma. Congenital causes of OA include cystic fibrosis, congenital absence of the vas deferens (CAVD), ejaculatory duct or prostatic cysts, and Young's syndrome [4]. NOA comprises a spectrum of testicular histopathology resulting from various

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Table 24.1 Glossary of terms in sperm processing

ICSI. Intracytoplasmic sperm injection: a procedure in which a single spermatozoon is injected into the oocyte cytoplasm
Azoospermia. Absence of spermatozoa in the microscopic examination of the seminal fluid after centrifugation on at least two separate occasions
PESA. Percutaneous epididymal sperm aspiration: a procedure in which a needle is inserted into the epididymis to retrieve spermatozoa for use in an ICSI procedure
MESA. Microsurgical epididymal sperm aspiration: a microsurgical procedure used to aspirate spermatozoa directly from the epididymal tubules for use in an ICSI procedure
TESA. Testicular sperm aspiration: a procedure in which a needle is inserted into the testis in order to retrieve spermatozoa for use in an ICSI procedure
TESE. Testicular sperm extraction: operative removal of testicular tissue in an attempt to collect sperm for use in an ICSI procedure
Micro-TESE. Microdissection testicular sperm extraction: 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
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
Cryopreservation. The freezing process for storage of gametes or gonadal tissue at ultra-low temperature

causes that include environmental toxins, medications, genetic and congenital abnormalities, varicocele, trauma, endocrinologic disorders, and idiopathic. Sperm can be easily obtained from men with OA whereas individuals exhibiting NOA have historically been the infertile men most difficult to treat [4, 5].

Several sperm retrieval methods have been developed to collect epididymal and testicular sperm in azoospermic men. It is out of our scope to discuss which technique is best to surgically retrieve sperm. As a general rule, either percutaneous (PESA) [6] or microsurgical epididymal sperm aspiration (MESA) [2] can be successfully used to retrieve sperm from the epididymis in men with OA. Testicular sperm aspiration (TESA) can be used to retrieve sperm from the testes either in men with OA who fail PESA or in those with NOA [6, 7]. Testicular sperm extraction (TESE) using single or multiple open biopsies [8–10] and, more recently, microsurgery (micro-TESE) are indicated for men with NOA [11–13] (Table 24.1).

Processing of surgically retrieved spermatozoa differs from the commonly used methods for processing ejaculates. 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 the handling of these often compromised specimens, particularly in the cases of NOA and after the freeze–thawing process. In order to achieve their goals, laboratory personnel should (1) receive the best quality surgically retrieved specimen possible, with minimal or no contaminants such as red blood cells and noxious microorganisms, (2) minimize the iatrogenic cellular damage during sperm processing by mastering technical skills and controlling several factors, including 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, and (3) improve the sperm fertilizing potential, if possible, by using stimulants or selecting viable sperm for ICSI when only immotile spermatozoa is available. In this chapter, our aims are to provide a step-by-step laboratory description of the commonly used methods for PESA/TESA/TESE sperm processing and identification of viable immotile sperm for ICSI, as well as to present our group's clinical results of ICSI using testicular and epididymal sperm. Finally, we critically discuss the tips and pitfalls of sperm processing techniques for surgically retrieved specimens.

Step-by-Step Protocol of the Laboratory Procedures

Materials, Equipments, Reagents, and IVF Laboratory Setup

Operating Room

- Sterile surgical gloves and syringes (1, 20 mL).
- 0.7 × 25 (22 G), 0.45 × 13 (26 G), and 1.2 × 40-mm (18 G; for TESA/TESE only) disposable needles.
- 2% Lidocaine hydrochloride solution.
- Heating block for test tubes (e.g.: Fisher, USA).
- Syringe holder (for TESA only, see Fig. 24.1).
- Operating microscope (e.g.: DF Vasconcelos, Brazil; for micro-TESE only).
- Microsurgery instruments (e.g.: ASSI, USA; for micro-TESE only).

IVF Laboratory

- 50 × 09-mm (e.g.: #351006, Falcon, USA) and 60 × 15-mm single well Petri dishes (e.g.: cat.#353037, Falcon, USA; for TESA/TESE only).
- Disposable serological pipettes (5.0 mL; e.g.: cat.#4051 Costar or cat.#356543 Falcon, USA).
- Pipettor 1–200 μL (e.g.: Gilson, France) and sterile tips (cat.#4804, Corning, USA).
- Pipetting device (e.g.: Pipette-aid, Drummond Scientific, USA).
- 6-mL sterile centrifuge polystyrene tubes with caps (e.g.: cat.#352003, Falcon, USA).
- 0.7 × 25-mm needles and tuberculin syringes (e.g.: BD, USA; for TESA/TESE only).

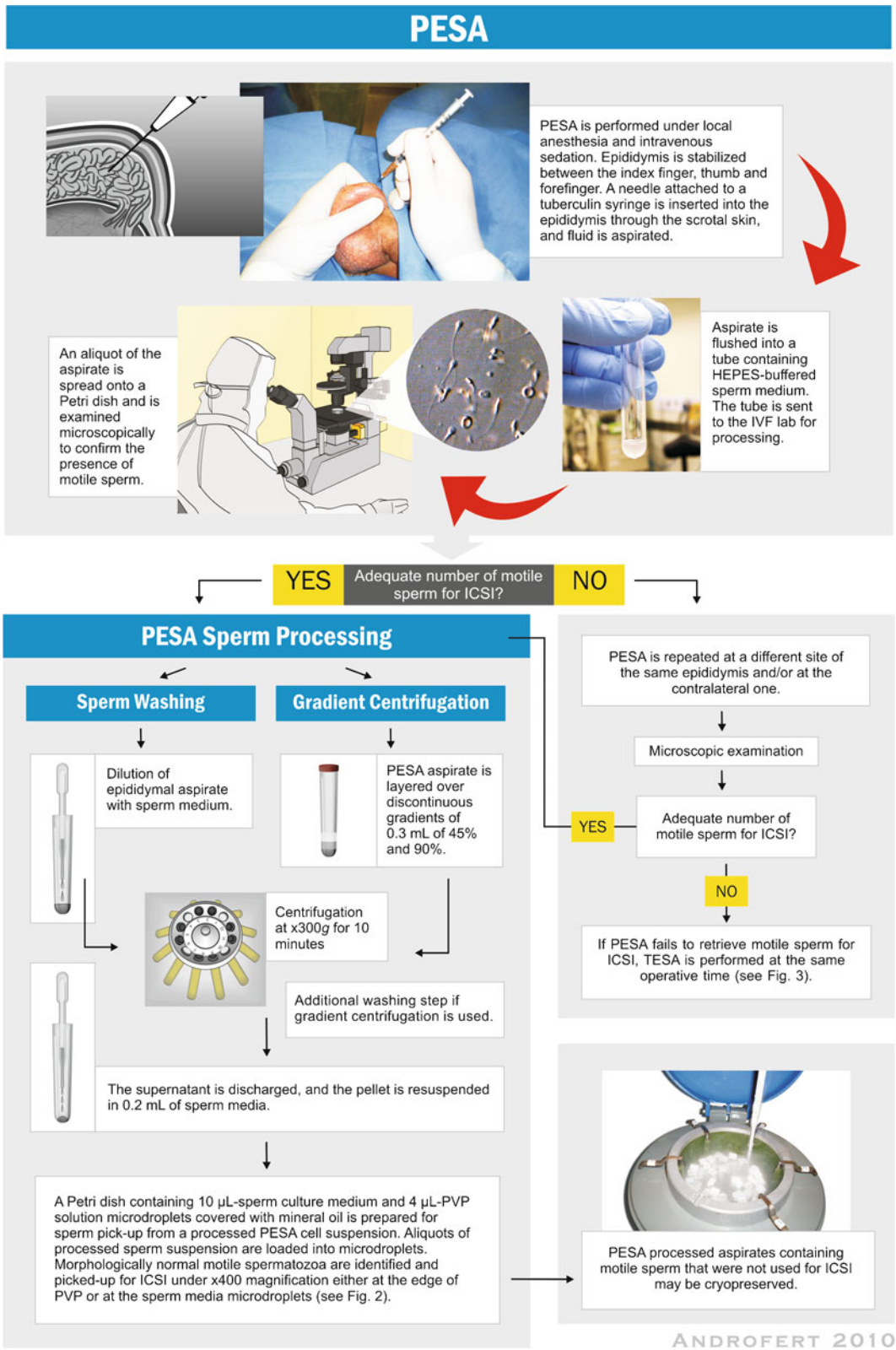

Fig. 24.1 PESA sperm processing. Flow chart illustrates PESA steps from the surgical procedure to the processing of epididymal aspirates for ICSI

- Fine point permanent marker pen (e.g.: Sharpie, Sandford, USA).
- Injection micropipettes (e.g.: cat.#MIC-50-35; Humagen, USA).
- Laminar flow cabinet (e.g.: Veco, Brazil).
- Warming plates (e.g.: Tokai-heat, Japan).
- Stereomicroscope (e.g.: Leica GZ7, Switzerland).
- Centrifuge (e.g.: model 225; Fisher Scientific, USA).
- Inverted microscope (e.g.: Eclipse E400, Nikon, Japan) equipped with Hoffman modulation contrast and electro-hydraulic micromanipulators (e.g.: Narishighe, Japan).
- HEPES-buffered Human Tubal Fluid (e.g.: modified HTF culture medium, cat.#90126, Irvine Scientific, USA) and human serum albumin (e.g.: HSA, cat.#9988, Irvine Scientific, USA).
- Mineral oil (e.g.: #9305, Irvine Scientific, USA).
- PVP solution (e.g.: cat.#10111, Vitrolife, Sweden).
- Colloidal density gradient (e.g.: Isolate®, cat.#99264, Irvine Scientific, USA).

Laboratory Setup

Note: Use sterile handling conditions under a laminar flow cabinet or clean room environment during all laboratory steps.

- Prepare a 10 (for PESA) or 20 mL (TESA/TESE) HEPES-buffered protein-supplemented (5% HSA) sperm culture medium, and keep it at 37°C.
- Transfer a 5 mL aliquot of the prepared sperm culture medium to a 6-mL polystyrene tube and send it to the operating room (sperm media is used to flush the aspirating system before aspiration and to incubate epididymal aspirates or testicular specimens upon collection).
- Place two 50×09-mm Petri dishes on a warm surface (37°C) inside the laboratory workstation (for PESA only).
- Prepare four 2-well dishes by transferring 0.5 and 1.0 mL sperm medium aliquots to the inner- and outer-dish wells, respectively (for TESA/TESE only). Place two of them onto a warm surface (37°C) inside the workstation, and send the others to the operating room (for TESE only).
- Mount two tuberculin syringes connected with a 22-gauge needle (to be used as tools for mincing and squeezing seminiferous tubules in TESA/TESE processing).

PESA Sperm Processing

Surgical Technique

Note: PESA is performed under local anesthesia (at the spermatic cord level) in association with intravenous anaesthesia using propofol either at the day of oocyte retrieval or the day before.

- 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 26-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. 24.1).
- 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 0.3–1.0 mL may be aspirated.
- The needle is withdrawn from the epididymis, and the aspirate is flushed into a 0.5–1.0 mL 37°C sperm medium.
- The tube containing the epididymal aspirate is transferred to the IVF lab next door. PESA is repeated at a different site of the same epididymis (from cauda to caput) and/or at the contralateral one until adequate number of motile sperm is retrieved. If PESA fails to retrieve motile sperm for ICSI, TESA is performed at the same operative time (Fig. 24.1).

Sperm Processing

- Homogenize epididymal aspirate and sperm medium to avoid sperm agglutination (epididymal spermatozoa tend to agglutinate fast). Keep aspirate-containing tubes capped at 37°C.
- Place a 10–20 μL sperm suspension aliquot onto a Petri dish, and spread it as thin as possible using a micropipette tip. Examine the fluid under the inverted microscope (×400 magnification) to confirm the presence of motile sperm. Inform the surgeon promptly if an adequate number of motile sperm is available for ICSI. This step should take no more than 2–3 min to allow the surgeon to decide on continuing or finishing the surgical retrieval. If more PESA specimens are taken, pool samples of similar quality together for processing. If PESA fails and TESA specimens are obtained, process specimens according to the "TESA processing protocol" (see section "TESA Sperm Processing" and Fig. 24.2).
- Upon finishing surgical retrieval, identify aspirate-containing tube(s) according to the epididymis side and site of aspiration, as well as to the presence of motile sperm. Make a decision upon the processing method to be used, i.e., simple washing or two-layer discontinuous mini-gradient centrifugation, based on a gross estimate of sperm density and motility. Use gradient centrifugation when the specimen contains high density of motile sperm, particularly if contaminated with red blood cells, cellular debris, and immotile sperm. Otherwise, use 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 $300 \times g$ for

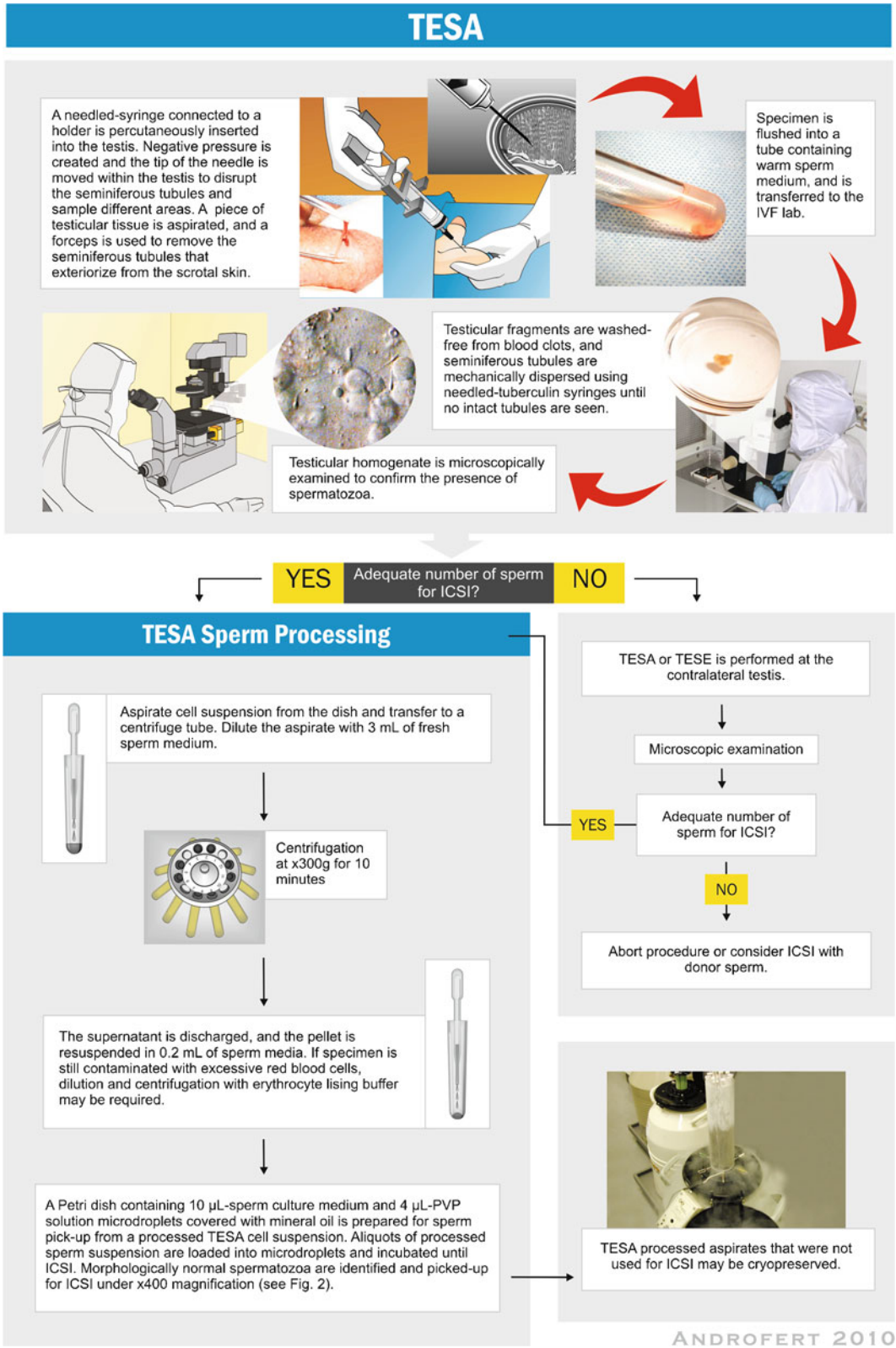

Fig. 24.2 TESA sperm processing. Flow chart illustrates TESA steps from the surgical procedure to the processing of testicular specimens for ICSI

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 medium above the pellet. Resuspend the pellet and keep at 37°C until use.

- For simple washing, dilute 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 then resuspend the pellet in 0.2 mL of sperm medium. When a processed PESA sample is still contaminated with an excessive number of red blood cells, dilution and centrifugation with 2 mL erythrocyte lysis buffer may be required (see Table 24.2).

Table 24.2 Solutions for washing samples

Erythrocyte lysis 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 [33]
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 [21]. Alternatively, a 139 mOsm/kg HOS solution can be prepared by mixing 1 mL sperm medium to 1 mL sterile reagent water [19, 20]
Pentoxifylline solution (PF): Prepare a 5-mM solution of PF by dissolving 1.391 mg pentoxifylline (Sigma cat. No. P-1784) in 1 mL of HEPES-buffered culture medium

- Prepare a Petri dish containing a series of microdrops under mineral oil for sperm pickup from a processed epididymal cell suspension (Fig. 24.3).
- Load gently a 1 μL sperm suspension aliquot at the center of the polyvinylpyrrolidone (PVP) microdroplet if the suspension contains motile sperm with progressive motility. After 5–20 min incubation period, morphologically normal motile spermatozoa can be identified and picked up for ICSI with the injection micropipette under ×400 magnification at the edge of the PVP droplet. If progressive motility is low or absent and/or the sample is contaminated with cellular debris, load 1–4 μL sperm suspension aliquot at each 10 μL peripheral microdroplet of HEPES-buffered culture medium to facilitate search and selection of motile sperm. First, aspirate a small volume of PVP into the injection micropipette to improve control during sperm pickup and to avoid blowing air bubbles during ejection of selected sperm into the PVP droplet.
- After finishing sperm pickup from the PESA-processed sample, wash the injection micropipette free of any debris in the PVP droplet.
- Make a final morphologic sperm assessment under ×800 magnification in the group of preselected spermatozoa before ICSI. Immobilize, aspirate into the micropipette, and inject selected sperm into the cytoplasm of metaphase II oocytes.
- Consider cryopreservation of leftover PESA-processed aspirates containing motile sperm that were not used for ICSI. Freezing can be carried out using the fast liquid nitrogen vapor method [14] (Fig. 24.1).

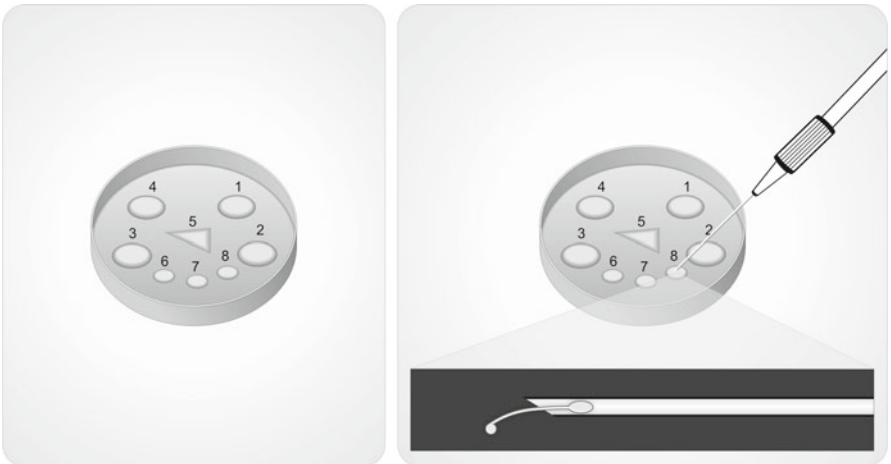

Fig. 24.3 Preparation of microdroplets. A 50 × 09-mm Petri dish containing several microdroplets of culture medium under mineral oil is prepared for sperm pickup from a processed epididymal or testicular cell suspension. Microdroplets are prepared as follows: four 10 μL sperm medium at dish periphery to load specimens (numbered 1–4), one 4 μL polyvinylpyrrolidone (PVP) at dish center in a triangle shape to pick up selected sperm for ICSI (number 5), and two to three 10 μL sperm medium at dish center below the PVP triangle for washing (numbered 6–8). Alternatively, one of the sperm medium-containing microdroplets (e.g., number 8) or the peripheric ones (1–4) may be replaced with the hyposmotic or motility stimulant solutions, respectively (*left*). The hyposmotic swelling test is illustrated (*right*). 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

TESA Sperm Processing

Surgical Technique

Note: TESA is performed under local anesthesia, as described in the "PESA protocol," in association with intravenous anaesthesia using propofol either at the day of oocyte retrieval or the day before.

- After anesthetic blockade of the spermatic cord, the epididymis is stabilized between the index finger, thumb, and forefinger while the anterior scrotal skin is stretched.
- A 18-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, in an oblique angle towards the medium and lower poles (Fig. 24.2). Loupe-magnification is used to avoid small vessels seen through the skin.
- Negative pressure is created by pulling the syringe holder while the tip of 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 (see Fig. 24.2).
- The specimen is flushed into a tube containing 0.5–1.0 mL warm sperm medium and is transferred to the IVF lab. TESA or TESE may be performed at the contralateral testis if insufficient number of sperm is retrieved for ICSI.

Sperm Processing

- Discharge TESA aspirate to the outer-dish well. Under stereomicroscopy, identify seminiferous tubules and remove blood clots using the needled tuberculin syringes.
- Transfer seminiferous tubules to the inner-dish well containing fresh sperm medium. Perform a mechanical dispersion of the tubules by mincing repeatedly 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 (see Fig. 24.2).
- 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 sperm for ICSI is available. This step should take no more than 10 min because the patient is kept under anesthesia until a decision of continuing or finishing the surgical retrieval is made. If other TESA specimens are taken, carry out the initial processing steps described above. If TESE specimens are obtained, perform processing according to the "TESE sperm processing protocol" (see section "TESE Sperm Processing" and Fig. 24.4).
- Aspirate and transfer the cell suspension from the inner-well dish 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. When a processed TESA specimen is still contaminated with an excessive number of red blood cells, dilution and centrifugation with erythrocyte lysis buffer may be required (see Table 24.2).
- Prepare a Petri dish as described in the "PESA protocol" for sperm pickup from a processed testicular cell suspension (see Fig. 24.3).
- Load 1–2 μL sperm suspension aliquot at each 10 μL peripheral microdroplet of HEPES-buffered culture medium to facilitate sperm search and pickup. Proceed to sperm selection and ICSI, as described in the "PESA protocol," and consider cryopreservation of leftover testicular aspirates. Dishes with microdroplets containing TESA-processed sperm can be incubated up to 48 h before ICSI at room temperature in an attempt to improve testicular sperm motility.

TESE Sperm Processing

Micro-TESE Surgical Technique

Note: TESE is performed under local anesthesia, as described in the "PESA protocol," in association with intravenous anaesthesia using propofol either at the day of oocyte retrieval or the day before. For micro-TESE, operating microscope and microsurgery technique are used throughout the procedure, as previously described [5, 11] (Fig. 24.4).

- After anesthetic blockade of 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 anesthetized layers, and the testis is exteriorized.
- A single, large, midportion 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 (more likely to contain germ cells and eventually normal sperm production). The superficial and deep testicular regions may be examined, if necessary, and microsurgical-guided testicular biopsies are performed by removing enlarged tubules (Fig. 24.4). 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 microbiopsies (at least three at each testicular pole) are performed.

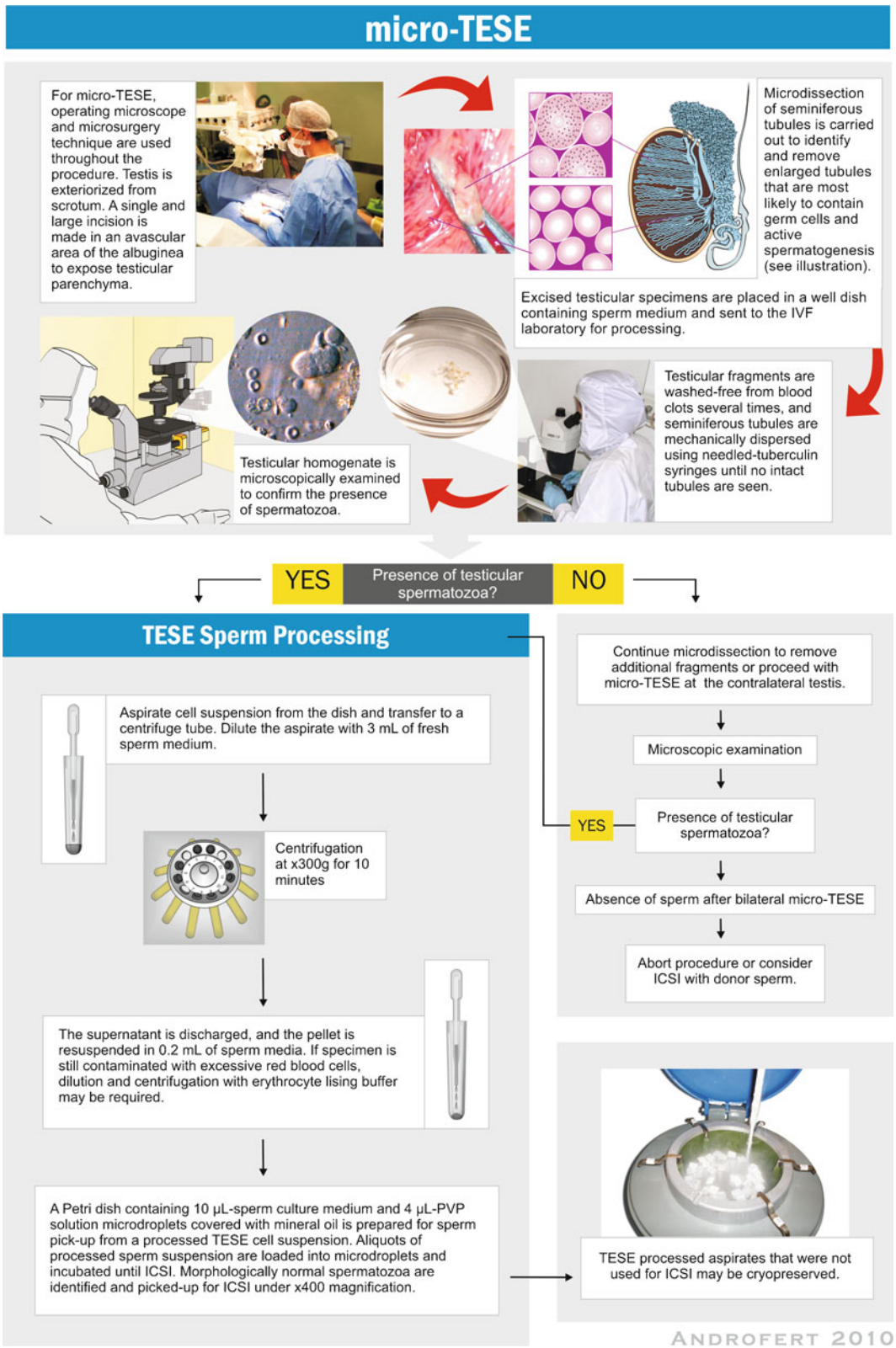

Fig. 24.4 TESE sperm processing. Flow chart illustrates TESE steps from the surgical procedure using micro-TESE for the extraction of testicular parenchyma to the processing of testicular specimens for ICSI

- Each excised testicular tissue specimen is placed at the outer-well dish containing sperm media. Specimens are washed grossly to remove blood clots and are sent to the IVF laboratory for processing.

Sperm Processing

- Transfer TESE fragments from the operating room dishes to the outer-well of a new dish in the IVF lab. Under stereomicroscopy, remove blood clots using the needled tuberculin syringes. Transfer fragments to the inner-well dish containing fresh medium and wash again until no blood clots are seen. Repeat these steps using new dishes if necessary, and make sure to start the mincing steps only when erythrocyte contamination is minimum (TESE fragments tend to be contaminated with excessive red blood cells).
- Perform mechanical dispersion of the tubules, and follow the steps described in the "TESA protocol" (Fig. 24.4). Two laboratory technicians/embryologists should 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). Inform the surgeon promptly if any sperm is found to allow him to decide upon continuing testicular microdissection or moving to the contralateral testis. If other TESE specimens are taken, carry out the initial processing steps described above.

Cryo-Thawed Epididymal/Testicular Sperm Processing

Epididymal and testicular spermatozoa may be cryopreserved using protocols routinely used for ejaculated sperm [15, 16]. After thawing, removal of cryoprotectant is carried out by simple washing, as described in the "TESA sperm processing protocol." If only immotile spermatozoa are seen, a method for selecting viable sperm for ICSI may be used.

Methods for Selecting Viable Immotile Sperm for ICSI

It has been observed that conventional seminal parameters have little or no influence in ICSI outcomes, except when only immotile spermatozoa are available [17, 18]. In certain cases, only immotile spermatozoa are obtained after fresh or cryo-thawed PESA/TESA/TESE processing. Different strategies are described to differentiate live immotile spermatozoa from dead ones, thus aiding in the selection of viable gametes for ICSI.

Hyposmotic Swelling Test (HOST) [19–21]

- 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 to the hyposmotic solution (HOS) microdrop (see Fig. 24.3 and Table 24.2), 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 [21]).
- 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.

Sperm Tail Flexibility Test (STFT) [22, 23]

- Using the microinjection pipette, pick up morphologically normal immotile spermatozoa from sperm microdroplet (see Fig. 24.3) and transfer to PVP solution.
- Align 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 [22, 23]). If tail remains rigid upon touching and sperm head and tail move together as a unit, the spermatozoon is then considered nonviable for ICSI.
- Repeat these steps until sufficient number of viable sperm is selected for ICSI.

Motility Stimulant Sperm Challenge (MSC) [24–26]

Note: Example given using a 5-mM pentoxifylline (PF) solution (see Table 24.2).

- Load a 4 μL aliquot of fresh or cryopreserved PESA/TESA/TESE sperm suspension into the motility stimulant solution microdroplet, and incubate for 20 min (see Fig. 24.3).
- Examine the specimen microscopically searching for moving sperm. In cases of positive MSC, 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× to wash out any residual PF solution (PF was shown to be embryotoxic in animal studies [27], but is apparently safe if used only on sperm [26]).

- Keep selected spermatozoa in culture or place them into a PVP droplet for sperm selection and immobilization before ICSI.
- Repeat these steps until sufficient number of viable sperm is selected for ICSI to be carried out.

Expert Commentary

PESA and TESA are effective surgical sperm retrieval methods for men with OA regardless of the cause (Table 24.3). However, the adoption of strict criteria to determine that the azoospermia is indeed obstructive is crucial for obtaining a high successful retrieval rate in the range of 90–100%. Using PESA, our approach is to perform the first aspiration at the epididymis corpus, and proceed to the caput if necessary as aspirates from the cauda are usually rich in poor-quality senescent spermatozoa, debris, and macrophages. Based on our findings, motile spermatozoa is obtained in 73% of the cases after the first or second aspiration. TESA is required as a rescue procedure after a failed PESA in approximately 14% of the individuals (Table 24.3). Most cases of PESA failures are not necessarily technical failures because immotile spermatozoa are found. However, in certain cases of epididymis fibrosis due to multiple PESA attempts or postinfection, PESA may be ineffective. In these cases, PESA can be attempted at the contralateral epididymis or TESA can be applied (Table 24.3). Routinely, procedures are performed under local anesthesia, with or without intravenous sedation, and at the same day of oocyte retrieval. 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 hematoma, bleeding, and infection, but are rare [8]. Some authors advocate that MESA allows the collection of larger and cleaner quantities of sperm than PESA, but this debate is trivial. In our series of 142 men with OA, cumulative successful retrieval rate after PESA and/or TESA was 97.9% (Table 24.3), and an adequate number of motile sperm for cryopreservation was obtained in approximately 31% (35/112) of the cases. Clinical outcomes of ICSI using PESA- or TESA-derived spermatozoa are similar in OA (Table 24.4), and results are comparable to those obtained with ejaculated sperm [28] (Table 24.5). Although the cryopreservation rate after PESA is not high, repeated aspirations can be carried out in men with OA at lower cost and morbidity than MESA. In rare circumstances, we perform MESA in men presenting coagulopathies.

When gradient centrifugation is chosen for PESA sperm processing, we recommend that part of the sample is spared and processed by simple washing. The reason is the unpredictability of gradient centrifugation to recover motile sperm in such cases. If recovery is less than desired, we can 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.

For NOA, TESE, with or without magnification, is clearly the preferred approach. Efficiency of TESA for retrieving spermatozoa in NOA is only 10–30% [29], except in the favorable cases of men with testicular histopathology showing hypospermatogenesis, to whom retrieval rates are approximately 80–100% [13] (Table 24.6). Nonetheless, if a previous TESA attempt had been successful in a man with

Table 24.3 Sperm retrieval rates (SRR) of PESA and TESA in obstructive azoospermia (AO), according to the number of aspiration attempts and the cause of azoospermia

	Presence of motile sperm by PESA; *N* (%)	Cumulative successful retrieval rate; *N* (%)
By retrieval attempt; *N* (%)—130 (100.0%)		
First PESA attempt	66/130 (50.8)	–
Second PESA attempt	29/64 (45.3)	95/130 (73.1)
Third PESA attempt	10/35 (28.6)	105/130 (80.7)
Fourth PESA attempt	7/25 (28.0)	112/130 (86.1)
Rescue TESA after failed PESA	16/18 (88.9)	128/130 (98.4)
By subtype of OA; *N* (%)—142 (100.0%)[a]		
Congenital—30 (23.1%)	21/30 (70.0)	30/30 (100.0)[a]
Vasectomy/failed reversal—64 (49.2%)	40/64 (62.5)	61/64 (95.3)[a]
Postinfection/trauma/iatrogenic—48 (36.9%)	31/36 (81.6)	48/48 (100.0)[a]
Overall SRR; *N* (%)	92/130 (70.8)	139/142 (97.9)[a]

Source: Androfert, Center for Male Reproduction
Congenital: includes cases of CAVD (congenital absence of vas deferens) and ejaculatory duct obstruction (EDO)
[a]PESA+TESA: In 12 cases of postinfectious OA, TESA was performed as the first choice due to intense epididymis fibrosis

Table 24.4 ICSI outcomes using fresh epididymal or testicular spermatozoa retrieved from men with AO

	Epididymal	Testicular	Combined[a]
Number of cycles; *N* = 136	93	14	29
Female age in years; mean ± SD	32.6 ± 5.3	32.1 ± 5.4	31.6 ± 7.4
2PN fertilization rate (%)	66.0	56.6	59.8
Cleavage rate (%)	99.4	95.7	98.5
Top-quality embryo rate for transfer[b] (%)	51.9	48.9	51.3
Cycles with embryo transfer; *N* (%)	88 (94.6)	14 (100.0)	29 (100.0)
Number of embryos transferred; mean	2.8	3.3	2.6
Clinical pregnancy rate per transfer; *N* (%)	45/88 (51.1)	7/14 (50.0)	13/29 (44.8)
Miscarriage rate; *N* (%)	11/45 (24.4)	1/7 (14.3)	3/13 (23.1)
Live birth rate per transfer; *N* (%)	34/88 (38.6)	6/14 (42.8)	10/29 (34.5)

Source: Androfert, Center for Male Reproduction
Values expressed as means for fertilization, cleavage, and embryo quality rates
[a]Oocytes were microinjected with epididymal or testicular spermatozoa retrieved from the same patient in the treatment cycle
[b]7–9 Blastomeres of similar size, and grades I or II cytoplasmic fragmentation on the day of embryo transfer (day 3)

Table 24.5 ICSI outcomes using spermatozoa derived from ejaculates and retrieved from the epididymis or the testis in men with obstructive and nonobstructive azoospermia

	Ejaculated sperm (*N* = 220)	Surgically retrieved sperm from azoospermic men (*N* = 93)		
		AO (*N* = 39)	NOA (*N* = 54)	*P* value
2PN fertilization rate (%)	70.0[a]	73.6[b]	52.2[c]	<0.05[c×a, b]
Cleavage rate (%)	91.0[a]	94.8[b]	77.7[c]	<0.05[c×a, b]
Top-quality embryo rate* (%)	48.5[a]	46.3[b]	35.7[c]	<0.05[c×a, b]
Clinical pregnancy rate (%)	43[a]	53[b]	26[c]	<0.05[c×a, b]
Miscarriage rate (%)	13	20	14	NS

Adapted from Verza and Esteves [28], with permission
Values expressed as means for fertilization, cleavage, and embryo quality rates. One-way ANOVA was used to compare clinical and laboratorial variables between groups, and the chi-square test was used to compare pregnancy and miscarriage rates. $P < 0.05$ was considered significant; *NS* not significant; Superscripts alphabets indicate pairwise group comparisons
OA obstructive azoospermia; *NOA* nonobstructive azoospermia
*7–9 Blastomeres of similar size, and grades I or II cytoplasm fragmentation on the day of embryo transfer (day 3)

NOA, its positive predictive value for a successful second attempt is 70% [30]. Based on these results, it is our opinion that TESA should be reserved for NOA individuals having a diagnostic testicular biopsy histopathology showing hypospermatogenesis or with a previous successful TESA attempt. However, if TESA fails, we neither perform a second aspiration in the same testis, at the same operative time, nor convert it to an open procedure, to avoid the risk of hematoma and testicular injury. From our clinical experience, it is very difficult to identify enlarged seminiferous tubules in such cases, even using the operating microscope, because extensive bleeding is often seen. In these occasions, we opt to perform TESA or TESE at the contralateral testis. For NOA patients without previous diagnostic testicular biopsy or TESA attempt, our choice is to perform sperm extraction using micro-TESE.

The use of optical magnification 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, the probability of retrieving sperm varies according to the testicular histopathology results [12, 13] (Table 24.6). Proper identification of testicular vessels under the tunica albuginea is made prior to the placement of an incision into the testis. Microsurgery also allows the preservation of intratesticular blood supply, as well as the identification of tubules more likely to harbor sperm production. 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 [31]. 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 pickup and injection process, as well as alleviates contamination

Table 24.6 SRR in 176 nonobstructive azoospermic men according to the surgical method of collection stratified by histopathology results and cause of azoospermia

	Presence of testicular spermatozoa; *N* (%)
By method; *N* = 176 (100.0%)	
TESA (*N* = 90); overall SRR, *N* (%)	58/90 (64.4)
Hypospermatogenesis	26/26 (100.0)
Maturation arrest	2/6 (33.3)
Sertoli cell only	6/29 (20.7)
Not available[a]	24/29 (82.3)
Micro-TESE (*N* = 86); overall SRR, *N* (%)	45/86 (52.3)
Hypospermatogenesis	19/19 (100.0)
Maturation arrest	7/12 (58.3)
Sertoli cell only	13/39 (33.3)
Not available	6/16 (37.5)
By cause of NOA; *N* = 176 (100.0%)	
Varicocele (66)	45/66 (68.2%)
Genetic (*n* = 12)	6/12 (50.0%)
Cryptorchidism (*n* = 19)	12/19 (63.1%)
Idiopathic (63)	33/63 (52.4%)
Radiotherapy/chemotherapy (*N* = 6)	3/6 (50.0%)
Orchitis/gonadotoxin/endocrine/other (*N* = 10)	10/10 (100.0%)
Overall SRR; *N* (%)	109/176 (61.9)

Source: Androfert, Center for Male Reproduction
[a]Cases with previous successful TESA attempt

and blockage of the injection needle with cells and debris. It is far less technically demanding and labor 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. TESE sperm processing may be incredibly labor intensive, and the searching process may miss the rare spermatozoa within a sea of seminiferous tubules and other cells. TESE/micro-TESE may be scheduled either for the day of oocyte collection and ICSI or the day before. In the latter, 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 room for a maximum of 48 h, to avoid bacterial contamination. Culture of specimens at 37°C inside the incubator should be avoided since contamination with scrotum skin-derived bacteria is often seen. 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 [32]. Testicular tissue sperm processing, searching, and selection of viable spermatozoa for ICSI may take several hours in NOA cases. Our laboratory takes approximately 12 min to handle a single testicular spermatozoon from searching 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 elect to perform micro-TESE the day before oocyte collection when a busy next day IVF laboratory workload is anticipated. Additionally, we recommend that 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 instead of using enzymatic digestion. The mechanical preparation has the advantage of being fast, requiring about 15–30 min, while enzymatic digestion is more time consuming, requiring at least 4 h [33]. Studies comparing both techniques show conflicting results, but large series were unable to confirm the superiority of one technique over the other for processing fresh or frozen testicular sperm [33–36].

The clinical outcomes of ICSI using testicular sperm extracted by TESA or micro-TESE from NOA individuals are significantly lower than those obtained with either ejaculated or epididymal/testicular sperm taken from men with OA [28] (Table 24.5). Our data indicate that testicular spermatozoa of men with severely impaired spermatogenesis have decreased fertility potential and may have a higher tendency to carry deficiencies such as the ones 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 normal zygote and a viable embryo [28].

The concept of cryopreservation may be used in association with sperm retrieval procedures. Some centers 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) at the same day. Also, cryopreservation may be an interesting tool to spare leftover specimens that would be discharged after ICSI, especially if the treatment cycle does not result in a pregnancy. Future ICSI attempts may be carried out without repeated surgical retrievals. We routinely freeze excess motile epididymal spermatozoa which are not needed for the current ICSI cycle. Most often, motile sperm will be available after thawing in such cases, and ICSI outcomes using motile fresh or frozen epididymal sperm seems not to differ [37]. Cryopreservation of testicular sperm is also advisable, especially for men with NOA who often require multiple ICSI attempts to conceive but may not have an adequate number of sperm available for repeated retrieval attempts. However, postthawed testicular sperm are often immotile or exhibit only a twitching motility, and ICSI results using immotile testicular sperm are lower than fresh ones [17, 38]. Methods for selecting immotile

viable sperm for ICSI are available, but results are limited for cryopreserved specimens. HOST may not discriminate viable and nonviable frozen–thawed spermatozoa [21]. Response to motility stimulants is unpredictable, and STFT has not been validated in large series [22–27]. 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 cryo-thawed specimens [39]. We currently use the STFT for discriminating viable and nonviable immotile sperm for ICSI. Using this method, normal fertilization rates obtained by our group has been fair (39.9%). In vitro incubation of fresh or frozen retrieved sperm in culture medium may aid to obtain a more viable and functionally normal sperm population that obviates the risks of using immotile unselected sperm for ICSI. Sperm culture media have the components to support normal metabolism of immotile, mature, retrieved spermatozoa that may become motile by incubation [40]. However, in vitro incubation should be limited to a maximum of 48 h since contamination by bacteria that normally come from the scrotal skin is almost a rule after a period of 36–48 h, even when strict sterile operating and laboratory conditions are used. According to each group's results, different strategies can be developed. If freezing of surgically retrieved specimens provides similar results than those obtained by using fresh sperm, then the use of freezing specimens would be preferable. If not, fresh specimens are preferable. In our hands, ICSI is performed with fresh testicular sperm whenever possible, even with the risk of submitting the female partner to unnecessary ovarian stimulation or the male to repeated retrievals. The reasons are related to the lower pregnancy rates obtained with cryothawed immotile testicular sperm by our group and others [18, 38, 40, 41]. To date, our technique for cryopreserve surgically-retrieved sperm is the standard liquid nitrogen vapor method using TEST-yolk buffer and glycerol as cryoprotectants [14–16]. Epididymal specimens are concentrated by washing before freezing, and testicular sperm are freed from the testicular parenchyma, i.e., testicular homogenates are frozen. Cryopreservation of few sperm into an empty zona pellucida and the use of stimulants before freezing may optimize results [15, 24, 42, 43]. Recently, it has been shown that human spermatozoa can be successfully vitrified, and this strategy may be of interest for preserving small quantities of surgically retrieved gametes [44].

Conclusions/Key Points

- PESA and TESA are simple and efficient surgical methods for epididymal and testicular sperm retrieval, respectively, in men with OA. For NOA, TESE, with or without magnification (micro-TESE), should be the preferred approach. The use of microsurgery during TESE may improve the efficacy of sperm extraction with significantly less tissue removed, which ultimately facilitates sperm processing. Sperm retrieval should be carried out either at the day of oocyte retrieval or the day before, depending on the laboratory workload.
- The primary goal of PESA/TESA/TESE sperm processing is the recovery of a clean sample containing motile sperm. Such specimens are more fragile, and often compromised in motility, as compared to the ones obtained from ejaculates. Laboratory techniques should be carried out with great caution to avoid jeopardizing the sperm fertilizing potential. The whole process starts with the surgical collection of the best quality specimen 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 equipments.
- PESA sperm processing may be performed either by mini-gradient centrifugation or simple washing using low volumes of culture media. Testicular specimens may be processed either by mechanical mincing of seminiferous tubules or enzymatic digestion, with similar results, and homogenates are simply washed. The mechanical preparation is significantly faster than enzymatic digestion.
- The use of unselected immotile epididymal/testicular sperm for ICSI negatively impact clinical outcomes. Methods for selecting viable immotile sperm for ICSI include the HOST, STFT, and motility stimulants.
- The concept of cryopreservation may be useful for intentionally cryopreserve retrieved sperm for future use or spare leftover specimens that would be discharged after ICSI. Different strategies can be developed according to each group's results. If freezing of surgically retrieved specimens provides similar results than those obtained with the use of fresh sperm, then the use of freezing specimens would be preferable. If not, fresh specimens are preferable.

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Processing Sperm Samples in HIV-Positive Patients

25

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Abstract

Human immunodeficiency virus is an important public health problem; about 40 million people around the world are actually infected with HIV type 1 virus. Furthermore, prolonged survival of patients under highly active antiretroviral therapy has given rise to a new demand from SDC couples who wish to have children. HIV serodiscordant couples with the male infected wish to make use of assisted reproduction techniques in combination with semen washing in order to decrease the risk of transmission to the partner or to the newborn. Thus it is essential to test the effectiveness of techniques aimed at reducing HIV and HCV loads in infected semen using molecular biology tests. Nevertheless, its efficiency is not absolute, and it remains unclear if the efficacy of sperm wash depends on the viral load of the ejaculate, in the laboratory handling or both. Our aim with this chapter is to compile the available information about sperm washes and assisted reproduction treatments with sperm samples obtained from serodiscordant males for HIV to describe the current possibilities of these males.

Keywords

Sperm • HIV • Assisted reproduction • PCR • Sperm sample processing • HIV and assisted reproduction

Prolonged survival of patients under HAART (highly active antiretroviral therapy) has given rise to a new demand from serodiscordant (SDC) couples who wish to have children. Unfortunately, due to the risk of transmission of viral particles through semen, the sexual intercourse in these couples should be done using protection, which eliminates any possibility to achieve pregnancies.

Thus, the only completely safe options available to these couples some years ago, to fulfill their 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 spermatozoa but also remove HIV-infected cells from the semen. These males need special assisted reproduction protocols in order to father children without risking transmission of their diseases to their partners and offspring [1], and these protocols mainly involve a treatment of the sperm sample and the confirmation of viral absence before any use.

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For HIV, there is a low transmissibility risk, established as 1–3 infections per thousand sexual intercourses (or semen exposures) [1]. At that time, all the accumulated data regarding viral transmission through semen with intrauterine insemination (IUI) is not enough to determine the real situation and must be revised. The employment of intracytoplasmic sperm injection (ICSI) on these couples is an applicable alternative to diminish this risk when using assisted reproduction technologies, 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 to the partner, offspring and laboratory staff during ART, we need to employ the safest methods available. To this end, we developed on SDC males, several strategies in the recent years to reduce the transmission risk to its lowest degree, maintaining reasonable and cost-effective pregnancy rates.

Our aim in this chapter is to present the reader with the information available regarding the safe employment of sperm cells for assisted reproduction in SDC couples with the male infected with HIV.

Sperm Wash and Detection of HIV-1 RNA and Proviral DNA Techniques

HIV belongs to the retroviruses family; they have the faculty to synthesize reverse transcriptase, convert the RNA form into DNA and to insert its genome in this manner into the hosts'. This issue will be very relevant for the detection methods employed [2], since we will need to demonstrate the absence of HIV in a sperm sample by analyzing both DNA and mRNA.

Several years ago, when washed sperm samples were employed for IUI treatments, immunofluorescence methods 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 the amplification of defined sequences of the viral nucleic acids [3, 4]. However, these methods have significant limitations, since the detection levels are 50–200 viral copies, thus not detecting viral presence below these thresholds.

Trying to improve the techniques available, we developed a protocol to reduce the detection limit to a single RNA or DNA viral copy with nested PCR (nPCR) or reverse transcription and nested PCR for RNA [5].

To this end, between August 2001 and October 2003, couples with HIV-positive males ($n=18$), couples with HCV-/HIV-positive males ($n=33$), and infertile couples undergoing ART where the male was HCV positive ($n=40$) were studied, yielding a total number of 134 ejaculates to be washed and analyzed. All of them signed an informed consent, and the study was approved by the Institutional Review Board. The mean age of all the men included in the study was 36.6 years old (range 25–47).

The HIV-infected patients exhibit all the stages of the infection (A, B, or C) according to the Center for Disease Control (CDC) classification. The mean viral load was 48,623 copies/mL (range 78–525,000 IU/mL), with 46 patients without detectable blood viral load. The mean CD_4 cell count was 502.7 CD_4/mm^3 (range 26–1,664). Finally, the time of evolution of the disease was 11 years (range 3–20).

Semen Analysis

All the samples must be processed in a dedicated laboratory; it is necessary to employ a highly trained and experienced staff and make use of an exclusive and isolated laboratory area and set apart from work with noninfectious samples; using class II/B3 biosafety cabinet, centrifuge with safety lids, an exclusive incubator and a nitrogen tank 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] criteria. Total 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-/HIV-positive samples, and, as these samples are always for ICSI treatments, fresh sperm morphology is not needed.

Sperm Wash Procedure

The sperm wash consisted of three steps (Fig. 25.1):

Step 1

- An initial dilution 1:1 (vol:vol) with Sperm Medium (MediCult, Jyllinge, Denmark), of the ejaculates after liquefaction.
- Then, they were pelleted at $400\times g$ for 10 min, and the supernatants were discarded.
- An equal volume of Sperm Medium as the initial volume of was added ("first wash").

Step 2

- Prepare 3–4 conic tubes of triple density gradients of 1–1.2 mL of each layer (90, 70, and 45%, PureSperm, Nidacon, Goteborg, Sweden) and layered 1 mL approximately of "the first wash" onto each tube.
- Centrifuged 20 min at $300\times g$ and then eliminate the three upper fractions (first wash, 45 and 70% layer) of each tube.

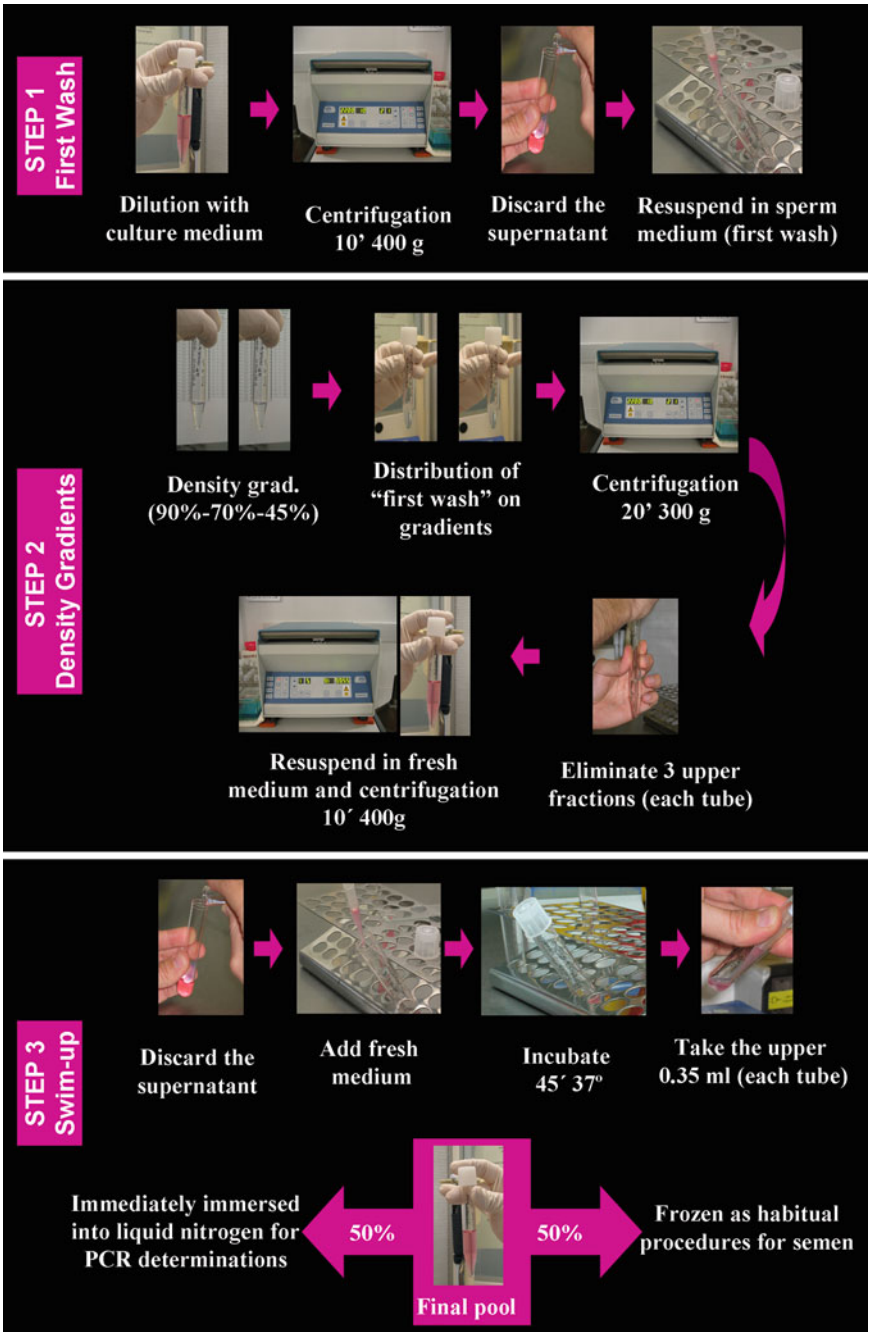

Fig. 25.1 The sperm wash procedure consists of three steps

- Each pellet was obtained and washed with 5 mL of Sperm Medium, and re-pelleted again at 400×*g* 10 min. Supernatants were discarded.

  Step 3
- After the supernatants were discarded, a swim up of 0.5–0.7 mL was done. After 45 min, the upper 0.35 mL of each tube was obtained and pooled.
- After evaluating the total count and motility, one half of a "pool" was immediately immersed into liquid nitrogen for PCR determinations, and the other half was frozen with Sperm Freezing Medium (MediCult, Jyllinge, Denmark), following the manufacturer's instructions, and stored until their use after a negative result of viral presence [5].

PCR Techniques for HIV Applied to Semen

Nucleic acids were extracted from the washed spermatozoa using the NucliSens (Organon-Teknika, Barcelona, Spain), following the instructions of the manufacturer. Two extractions were run in parallel, one from the spermatozoa sample

and the other with the spermatozoa samples after the 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 a nested DNA amplification [7]. The same samples were used to amplify HIV proviral DNA by a 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 negative controls to detect the presence of amplicons, contamination was carried out.

For HIV RNA transcription, we used the antisense external primers to anneal 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 each dNTP, 0.2 μM antisense primer, 20 U RNAasin (Promega, Barcelona, Spain), and 5 U AMV transcriptase (Promega) in a final volume of 20 μL. Nested DNA amplification used the external primers to anneal with nucleotides (from 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 [8].

PCR standardized 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 for each dNTP (Amersham Pharmacia, Madrid, Spain), 0.5 μM for each primer, and 2 U Taq polymerase (Promega) in a 50-μL final volume. β-actin (beta-actin) gene amplification was performed to confirm the presence of DNA in the extraction from spermatozoa suspension. Results were read after a 2% agarose gel electrophoresis after ethidium bromide staining [7]. In all 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 of Roche Diagnostic Systems (Amplicor; Roche, Basel, Switzerland) and as described in the work by Marina and coworkers [3]. Briefly, RNA extraction was performed according to the Amplicor Specimen Preparation Kit (Roche, Barcelona, Spain).

To check for 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 ELISA was used for subsequent detection.

The results of the analysis for the detection of the presence of viral molecules after the sperm washes demonstrated that approximately 7% of sperm washes still resulted positive after the sperm wash. On those cases, the sample was reanalyzed, and the results of positive samples by nested PCR were compared with the one-round PCR protocols, finding that they resulted negative.

After 2–3 weeks, another sperm wash is programmed. We never obtained a positive result after a second wash. The assay never failed in yielding a diagnostic.

This is undoubtedly indicating that a negative sample in the protocols employing one-round PCR still had undetectable viral presence [2]. Nevertheless, different institutions employ different methodologies, with highly variable efficiency and detection limits [9, 10].

Assisted Reproduction Techniques on Serodiscordant Couples

To choose which treatment (IUI, in vitro fertilization (IVF), or ICSI) would be used, several aspects must be considered. Artificial insemination has been the alternative method for a period of time, given the low difficulty and invasiveness [3, 4]. This method may be simpler and less expensive than others, but we think that it presents some important inconveniencies, such as the need of a sperm wash result on the same day, and if a positive result is found, the cycle will be canceled; another problem is the low numbers of spermatozoa inseminated and the potential risk of viral transmission due to the exposure to thousands or even millions of "potentially infecting" spermatozoa. Also the whole sperm preparation must be inseminated within the same cycle. In this way, pregnancy rates are about 15% per cycle, with accumulative pregnancy rates of almost 60% in four consecutive treatments depending of sperm quality (considering a significant decrease of the sperm number available after sperm wash process).

We maintain a different conduct with these couples: IVF with ICSI protocols. ICSI involves a lower exposure of sperm cells compared to that in IUI (which requires millions of sperm). Despite the high cost of the ICSI process; the efficiency of this technique avoids the 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 (two to three times higher with ICSI; nearly 50% per cycle, with cumulative pregnancy rates in three cycles of 90%) and should decrease the number of attempts needed to establish a successful pregnancy.

With ICSI, we are exposing the woman to a nearly infinitesimal risk: we just employ one single sperm for each oocyte obtained from a previously sample resulting negative by PCR.

Table 25.1 Global cycle results depending on the infection

	HIV (total)	HIV only	HIV/HCV	HCV infected
Follicular aspirations (FA)	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
Met. II/FA	9.9 ± 2.3	10.9 ± 2.0	10.2 ± 2.0	10.5 ± 3.0
Fertilization rate/Met. II (%)	68.9 ± 6.3	72.0[a] ± 8.1	66.8 ± 6.0	59.3[a] ± 5.3
Embryos/(FA)	4.3 ± 2.0	5.1 ± 1.8	4.0 ± 2.0	3.8 ± 0.7
Cleavage rate (embryos/Met. 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 (FE) transfer	10	6	4	8
FE positive pregnancy tests	2	0	2	0
FE negative pregnancy tests	8	6	2	6
FE pregnancy rates (%)	20	0	50	0

Results are expressed as total numbers, or mean ± standard error of the mean.
[a] $p < 0.05$

By another way, semen samples are frozen, and nested PCR results can be confirmed as many times as needed, since that the sample is not necessarily employed the same day. Also, sperm wash can be performed before the cycle, thus not canceling any cycle due to a positive result. If sperm wash resulted negative, it can be employed several times as needed, thus avoiding the need (and expenses) of new washes.

Additionally, in men with a priori lower semen qualities, that will not be enough for optimal IUI, ICSI is the only realistic treatment option because after the extensive procedure that involves sperm wash, and in order to avoid possible positives, 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 their reproductive wishes safely [3, 4, 11–15].

Sperm Wash Results: Efficiency, Pregnancy, and Seroconversion Rates

Next, we are going to analyze the results of our program for SDC couples with the male infected, in terms of seroconversion and pregnancy rates when washed samples were employed. Couples with seronegative women, and HIV-positive males ($n = 18$), with co-infection HCV-/HIV-positive males ($n = 33$), and infertile couples undergoing ART where the male was HCV positive ($n = 40$) were included in this study, providing a total number of 134 sperm samples to be washed. The HIV infection was acquired by parenteral drug addiction in 25 of them (48.8%), plasma donation in 1 (0.2%), sexual transmission in 11 (21.6%), blood transfusion in 6 (11.7%), and 8 (15.5%) unknown. About the female population, only those with demonstrated absence of HIV and HCV antibodies were accepted in the study. They were also requested to practice sex with condoms.

Different gynecological findings were observed: 59 of them were considered as normal (64.8%), 15 of them were aged more than 36 years old (16.4%), 5 of them were low responders (5.5%), 8 of them had endometriosis (8.6%). Depending of the patient's characteristics, we treated these women with ICSI with their own oocytes or with oocytes obtained from young healthy donors. In HIV serodifferent couples, 11 procedures were made with donated eggs, while seven procedures were performed with donated eggs in HCV serodiscordant couples.

In this work, we found a global pregnancy rate of approximately 45% per cycle [1], and almost 95% of the couples finally get pregnant after four consecutive cycles. Results are shown in Table 25.1. Blood analysis after 3, 6, and 9 months confirmed no seroconversion in the women.

Table 25.2 ROC curves analysis results for the prediction of the post-wash PCR result for infectious particles by semen parameters and HIV patient's features

Parameter ($n=73$)	Area under the curve	Threshold	Sensitivity	Specificity	VPP	VPN
CD_4 (HIV)	0.59 (0.469–0.704)	≤147	37.5	92.5	37.5	92.3
Evolution (HIV)	0.53 (0.410–0.653)	≤12	85.7	30.2	12.0	95.0
Viral load (HIV)	0.49 (0.289–0.690)	≤44,000	100	36.4	22.2	100
Volume	0.49 (0.397–0.576)	>3.4	61.5	55.2	13.3	92.8
Concentration	0.65 (0.561–0.731)	≤13.6	38.5	87.9	26.3	92.7
A+B	0.55 (0.458–0.636)	>67	23.1	93.9	30.0	91.5
TMP	0.62 (0.537–0.711)	≤38.07	69.2	73.9	23.1	95.5
Volume cap.	0.55 (0.463–0.642)	≤1.1	69.2	46.5	12.9	93
Conc. post	0.57 (0.479–0.658)	>1	100	20.4	12.6	100
A+B AW	0.59 (0.507–0.685)	≤80	69.2	57.5	15.8	94.2
TMP AW	0.68 (0.600–0.769)	≤2.2	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

A, *B* type A and B motility, respectively (WHO statements); *TMP* total motile progressive sperm; *AW* after wash

Influence of HIV-1 Infection on Semen Quality: The Basic Sperm Analysis and the Embryos Point of View

A posterior work was dedicated to the analysis of factors making the sperm washes fail and the sperm quality in SDC males. We evaluated male infection parameters, antiretroviral treatments received, and sperm quality to identify positive washes predictors [16].

In this study, 73 healthy seronegative males were included, as a control population, consisting in partners of women exclusively with tubaric infertility, within the same period of time. Controls and HIV males were matched by 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. Age ranged between 21 and 54 years (median 37.1)

The viral acquisition was for the 70 HIV-infected males (45 of them also co-infected with HCV, 64.3%); 32 ex-addictions to parenteral drugs (45.7%), 2 plasma donations (3.0%), 15 heterosexual transmissions (21.4%), and finally 14 (20.0%) were from unknown origin. The length of the HIV disease was 9.6 years (range 1–20). In the CDC classification of the disease, 12 of them (17.1%) were A1, 23 (32.8%) A2, 5 (7.1%) A3, 3 (4.3%) B1, 7 (10%) B2, 11 (15.7%) B3, 2 (2.8%) C2, and 6 (8.5%) C3.

About antiretroviral treatments, 21 of them (30%) were treatment-free, while the remaining were receiving any combination: 2 monotherapies (2.8%), 4 bi-therapies (5.7%), 36 (51.4%) tri-therapies, tetra-therapies (8.5%), and 1 hexa-therapy (1.4%).

HIV blood load was 19,408 IU/mL (ranging from undetectable-525,000 IU/mL), while 39 patients (55.7%) showed negative viral blood load. Peripheral blood CD_4 levels median was 497.5 ranging from 26 to 1,064.

The results of this work exposed that there is no system to predict a positive result after sperm washes, as stated by the ROC curves analysis of the different factors (Table 25.2). As well, we did not found a no significant diminish in sperm quality on HIV-positive group in comparison with healthy controls, and antiretroviral treatments received are not affecting sperm quality. Another way to analyze sperm quality is by studying the embryo quality obtained from these sperm samples in comparison with healthy controls [17].

From our data, we conclude that fertilization and cleavage rates were comparable between the groups. On days 2 and 3 of embryo development, very similar embryo features were found between the groups. Additionally, there was no difference in mean number of optimal embryos on day 3; but when embryos were cultured up to 5–6 days, a significant increase on embryo blockage was found in SDC group, in comparison with control group. However, the mean number of optimal blastocysts on day 6 was comparable in both groups. Regarding number of cryopreserved and transferred embryos, implantation, pregnancy, multiple pregnancy, and miscarriage rates, difference was found between the groups.

Is Sperm Wash Possible in Oligo- or Azoospermic Samples?

To undergo these sperm wash protocols, a minimum number of motile sperm cells in the ejaculate are necessary. At least two millions are needed to perform these treatments.

Patients with Severe Oligospermia

In our institution, there are approximately 2% of the HIV- or HCV-seropositive men presenting severe oligospermia [1].

On them, the exhaustive sperm wash protocols, executed as previously described, made impossible to recover any sperm at the end of the procedure.

On males that present lower sperm count, we considered the possibility to perform modified sperm washed by serial dilutions of the sperm sample and, then, to confirm the absence of viral nucleic acids by PCR.

The first series were published in 2006, where from 7 males with severe oligospermia undergoing modified sperm washes, none resulted positive and were able to undergo assisted reproduction treatments [18]. This is, to our knowledge, the unique report from oligospermic males.

Sperm Wash Protocol on Oligozoospermic Males

Ejaculates obtained after a sexual abstinence of 3–5 days should be allowed to liquefy for 10 min at 37°C and then be diluted 1:1 (vol/vol) with human tubal fluid (HTF) medium. Then, centrifuge for 10 min at 400 × *g*, and discard the supernatants carefully. This procedure has to be repeated twice, and the resulting cells will be resuspended in a final volume of 0.5–1 mL.

One half of each sample have to be submerged immediately in liquid nitrogen for PCR determinations, and the other half be frozen as described elsewhere [2] and be stored until its usage in assisted reproduction once a negative viral presence had been confirmed.

Patients with Azoospermia

An extreme situation is presented in males without sperm in the ejaculates. Two case reports were available in the literature describing the epididymal search of motile sperm in azoospermic males presenting obstructions in the genital tract.

On these papers, only patients with epididymal sperm retrieval were presented [19, 20]. 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 when males presenting nonobstructive azoospermia with no spermatozoa available in the epididymal reservoir.

Furthermore, open testicular biopsies augment the chances to find motile sperm cells in azoospermic patients, although the blood and round cell presence could be increasing the risk of viral presence after wash.

Our group recently published three cases with open testicular biopsy, in azoospermia, that is also applicable to males presenting nonobstructive azoospermia [21].

Sperm Wash Protocol on Azoospermic Males

Samples were obtained from male partners by testicular sperm extraction (TESE), due to the absence of sperm in two consecutive sperm analysis, obtained and analyzed from August 2007 to November 2008. All patients had normal karyotype and no microdeletions on the Y chromosome, as expected given the causes of azoospermia.

TESE was performed as previously described [22]. Briefly, open testicular biopsies were carried out with 2% mepivacaine spermatic cord block. After opening the scrotal skin and tunica vaginalis, 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 embedded within Bouin's solution and were sent for histopathological examination. The remaining fragments were carried to the adjacent laboratory for sperm retrieval. Testicular tissue was placed in 2 mL of Sperm medium (MediCult, Jyllinge, Norway) and minced mechanically with sterile slides. The presence of sperm cells was checked under an inverted microscope at 3,400 magnification. If motile sperm were found, samples were immediately frozen. Before that, they were centrifuged for 10 min at 400 × *g*, and the supernatants were then carefully discarded. 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 and 5% CO_2 for 1 h. Then, the presence of motile sperm was checked again.

Continually, for freezing, an equal volume of Sperm Freezing Medium (MediCult) containing glycerol was added to the sperm pellet and then homogenized and placed at room temperature for 10 min. Sperm samples were frozen in small tablets using a dry ice surface for approximately 1 min and then were transferred to pre-labeled 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 into liquid nitrogen tanks until its employment in assisted reproduction once a negative viral presence had been confirmed.

For thawing, the pills were removed and transferred into 5-mL Falcon tubes, and placed in an incubator at 37°C and 5% CO_2. Then samples were washed again with Sperm Medium (MediCult) and centrifuged at 600 × *g* for 5 min. The supernatant was discarded and samples resuspended in variable amounts of medium. Thereafter, motile spermatozoa were checked again for ICSI.

After sperm washes, motile sperm were recovered to be frozen in all testicular biopsies, then guaranteeing a safe use of the spermatozoa found within the sample. Two pregnancies were achieved in five follicular aspirations, with four fresh and two frozen/thawed embryo transferences, resulting in a healthy newborn and an ongoing pregnancy.

Our results, although in a limited sequence of cases, confirm that TESE-ICSI treatments in azoospermic seropositive patients are possible, and that these males should not be rejected if these techniques, together with sperm wash and PCR confirmation of the viral absence, are available

Conclusion

The laboratory is focused on two aspects: first, we need to eliminate any viral particle, since 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. Secondly, we must optimize the sperm washing procedure to ensure that we maintain as many motile sperm as possible, to permit as many ICSI procedures as possible.

In conclusion, HIV-infected patients now have the possibility to become fathers, avoiding viral transmission to the mother and future child. Sperm parameters are not significantly different from those that the World Health Organization considers to be normal. We were unable to detect a parameter that could help us in the prediction of the sperm wash results, which remained positive in 10% of the samples.

Low CD_4 counts together with a long time of evolution of the disease are relevant for the sample, but insignificant for the whole treatment. Given these results, patients must be counseled accordingly before the initiation of a treatment.

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Intracytoplasmic Morphologically Selected Sperm Injection

26

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Abstract

Intracytoplasmic morphologically selected sperm injection (IMSI) is now a reality in ART practice but still with a lot of questions regarding (1) the terminology of vacuoles, their classification, their location on the sperm head, and their origin and meaning; (2) the application of IMSI instead of in vitro fertilization in cases of unexplained infertility; (3) the age of the woman; and (4) the technical aspects. We have to be aware that this technique is demanding and has to be performed in the best working conditions so as not to impair the oocyte quality. The introduction of IMSI has the advantage that embryologists realize that more attention has to be paid during sperm selection even in case of classical intracytoplasmic sperm injection (ICSI). The application of IMSI leads to more and better quality blastocysts and, as consequence, it increases the chances of selecting the proper embryo for transfer with high implantation potential. Even though there is no real proof in the human species on the abnormal outcome generated by spermatozoa carrying vacuoles, a higher and better-resolution technique has to be added as an additional tool for ICSI knowing the possible consequence of sperm DNA damage for offsprings.

Keywords

Sperm injection • Intracytoplasmic sperm injection • Morphologically selected sperm • In vitro fertilization • ICSI • Sperm morphology

Since the beginning of in vitro fertilization (IVF), improvement in the stimulation protocols, in methods and strategies to select the best oocytes and embryos, in embryo culture media and protocol and in optimal preparation of the luteal phase are the main purposes of several studies and reports. Interestingly, since the introduction of intracytoplasmic sperm injection (ICSI), little attention has been paid to the spermatozoa. What is more, after the introduction of ICSI, several studies [1–3] find no relationship between ICSI outcomes with abnormal or normal sperm morphology. Such observations were probably biased by the selection performed by the embryologist who tried to select the best "normal-looking" motile spermatozoa prior to ICSI, which does not always reflect the quality of the whole semen population.

The evaluation of sperm morphology as a routine diagnostic control seems to be a powerful indicator of man's fertilizing potential in vitro and in vivo. The basic morphological evaluation (spermocytogram) is based on the WHO (World Health Organization) criteria [4] or Kruger's strict criteria [5] on ejaculated sperm after fixation and staining at

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magnification of ×1,000. Other powerful techniques such as transmission or scanning electronic microscopy permit a more accurate observation of nuclear defects or tail abnormalities (presence or absence of dynein arms) of spermatozoa. But, we have to recognize that it is impossible to select living morphologically normal spermatozoa with all those techniques requiring a fixation—staining step.

At the present, it is mostly believed that the success rate of ICSI mainly depends on morphological aspects of injected spermatozoa. De Vos et al. [6] observed a negative correlation with fertilization rates, ongoing pregnancies and implantation rates after ICSI, with occurrence of spermatozoa exhibiting elongated, tapered or amorphous heads, broken necks or cytoplasmic droplets.

As consequence, a more critical selection process of spermatozoa seems necessary.

During the last decade, there has been a growing interest on the development of new techniques that improve the preparation of the semen sample such as electrophoresis [7] or magnetic-activated cell sorting (MACS) [8]. Other techniques based either on a biochemical assay (hyaluronan binding assay) [9] or on high-magnification microscopical examination [10] were introduced to permit a better selection of those spermatozoa that can support embryo development to the birth of a healthy baby.

In this chapter, we will focus on a technique that allows us to assess the morphology of motile spermatozoa in real time before intracytoplasmic oocyte injection. The outcome of nuclear defect such as vacuole will be discussed.

Real-Time Morphological Approach: MSOME

The introduction of a new concept called "motile sperm organelle morphology examination" (MSOME) permits to examine the fine nuclear morphology of motile spermatozoa in real time, at a magnification of ×1,000 using Nomarski differential interference contrast optics [10, 11].

Morphological assessment at magnification up to more than ×10,000 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. At identical magnification, the use of Nomarski differential interference contrast optics lets us observe more precisely the general morphology of the spermatozoa and, in addition, minimal structural defects in the head, as compared with the conventional Hoffman modulation contrast.

MSOME in Combination with ICSI: IMSI

The MSOME approach in observing spermatozoa was then considered as an additional tool to ICSI and takes the name "intracytoplasmic morphologically selected sperm injection" (IMSI).

Morphological Normalcy of Spermatozoa Assessed by MSOME

Bartoov et al. [10, 11] define the morphological normalcy on motile sperm nucleus according to the shape and chromatin content. The shape has to be smooth, symmetric and oval with an average length and width limits estimated to 4.75 ± 0.28 and 3.28 ± 0.20 µm, respectively. The chromatin mass has to be homogeneous and contain 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.

Spermatozoa Selection and Reproductive Outcomes

IMSI with Morphologically Normal Spermatozoa

Fascinating results, showing the importance of the sperm morphology, were published immediately after the introduction of IMSI.

Bartoov et al. [11] stressed that among the group of subcellular organelles (head, tail, midpiece) observed by MSOME, the morphological normalcy of the sperm nucleus could be a crucial characteristic associated with a positive ICSI outcome.

They provided a first answer by performing MSOME on a leftover fraction of motile spermatozoa of 100 random couples referred for ICSI treatment. Among the different morphological aspects of the spermatozoa, the normalcy of the sperm nucleus (shape and chromatin content) was significantly related with fertilization, and no clinical pregnancy was achieved when less than 20% of the spermatozoa exhibit morphological normal nuclei.

Several publications report that selection of spermatozoa exhibiting normal nuclear shapes is positively associated with IVF outcomes after day 3 embryo transfers in couples with previous and repeated implantation failures [12–15] and in patients with an elevated degree of DNA-fragmented spermatozoa [16].

In a comparative prospective study, Bartoov et al. [12] compared the outcome of 50 couples that underwent IMSI with a group of 50 ICSI candidates after at least two previous failures of implantation and a maternal age of less than 37 year. The rate of good-quality embryos on day 3 (45.2% vs. 31.0%), the implantation (27.9% vs. 9.5%) and pregnancy rates (26.4% vs. 15.3%) were significantly higher after MSOME selection followed by injection of a morphologically normal spermatozoa.

Significantly lower abortion rates were observed after application of IMSI (9.0% vs. 33.0%).

Hazout et al. [16] perform a similar trial on 125 couples with previous failures of implantation and in addition with men exhibiting different levels of DNA fragmentation. Their study stressed the advantage of MSOME selection in terms of clinical pregnancy (2.4% vs. 37.6%) and implantation (0.8% vs. 20.3%) rates and this independent of the rate of DNA fragmentation.

In a recent study, Berkovitz et al. [14] reinforced the power of performing IMSI with morphologically normal spermatozoa in a group of patients with at least two previous failures of implantation. After comparing 80 ICSI trials with 80 matched IMSI trials, their conclusions corroborate their previous studies [12]: an increase in the rate of embryo quality on day 3 (25.7% vs. 38.7%), in the clinical pregnancy (25% vs. 60%) and implantation (9.4% vs. 31.3%) rates and a reduction in the abortion rate in the IMSI group.

IMSI with Morphologically Abnormal Spermatozoa

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 noticed already earlier by Berkovitz et al. [13, 14], 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.

Establishment of a Sperm Classification Using the MSOME Approach

As described in the previous chapter, Bartoov et al. [11] defined the morphological normalcy of the sperm nucleus according to the shape and chromatin content.

In the circumstances where no normal spermatozoa can be found, the only alternative consists then to select those that are morphologically second best. This meticulous approach towards sperm selection with the Nomarski differential interference contrast optics allows us to identify vacuole(s) in the sperm head that are otherwise not evident to detect at ×400 magnification with Hoffman modulation contrast.

It is then essential to know from the second choice spermatozoa with vacuoles and/or abnormal shape which one we have to select. For this reason, it appears that the establishment of a classification of the spermatozoa according to different types of abnormalities was necessary to select the spermatozoa in a more tangible way.

In order to know exactly the impact of specific sperm defects on embryo development and further outcome, Vanderzwalmen et al. [17] and Cassuto et al. [18] established a classification. The final aim was to classify individually the spermatozoon before injection combined with single embryo culture to day 5. With such an approach, they were able to compare and search for a relation between embryo development and the morphology of injected spermatozoa.

Vanderzwalmen et al. [17] classified the spermatozoa into four groups according to the presence and size of vacuoles: grade I, normal shape and absence of vacuoles; grade II, maximum of two small vacuoles; grade III, more than two small vacuoles or at least one large vacuole; and grade IV, large vacuoles in conjunction with abnormal head shapes or other abnormalities at the level of the base.

Cassuto et al. [18] established a detailed classification scoring scale ranging between 6 and 0 points according to the normalcy of the head (2 points if normal), the symmetry of the base (1 point if normal) and the absence of vacuole (3 points if absent). Three classes of scoring were established as follows: class 1, high-quality spermatozoa with calculated score of 4–6; class 2, medium-quality spermatozoa with calculated score of 1–3; and class 3, low-quality spermatozoa with calculated score of 0.

Effect of Injecting Morphologically Abnormal Spermatozoa on Reproductive Outcomes

Spermatozoa with unclassified defects. The importance of selecting normal spermatozoa is reinforced when comparing the reproductive outcomes in terms of fertilization, embryo development, pregnancy and abortions rates when oocyte injections are done with morphologically normal sperms and spermatozoa exhibiting different defects.

Berkovitz et al. [13] compared the IMSI outcome in two groups of 38 patients in which oocytes were injected with normal sperm or with spermatozoa exhibiting nucleus defects. They reported low fertilization rates (50.3% vs. 71.3%), low percentage of top-quality embryos on day 3 (19.4% vs. 34.9%) and low pregnancy (18.4% vs. 52.6%) and implantation rates (5.9% vs. 25.0%) after IMSI with spermatozoa exhibiting a large panel of nuclear malformations in terms of shape, size and the presence of vacuoles. The reproductive outcomes from this study were confirmed in a second larger study from the same group [14] comparing the reproductive outcome in two groups of respectively 70 patients injected with morphologically normal spermatozoa or second quality spermatozoa exhibiting defects.

Nuclear vacuoles. One particular sperm malformation that is better detectable (i.e. more precisely and easily) with the Nomarski optics is/are vacuole(s). Already in the early 1990s,

their presence has been negatively associated with natural male fertility potential [19, 20]. The potential relationship between large vacuoles and late embryonic developmental effects raises concerns.

A more specific analysis on the impact of sperm cells with normal nuclear shape but large vacuoles was first carried out by Berkovitz et al. [21] on two matched IMSI groups of 28 patients each. Spermatozoa with strictly defined normal nuclear shape but large vacuoles were selected for injection and compared to a control group that included normal nuclear shape spermatozoa lacking vacuoles.

No difference in the fertilization and early embryo development up to day 3 was 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%).

In two successive studies, Vanderzwalmen et al. [17] and Cassuto et al. [18] reported that the existence of large vacuoles in the nuclei of spermatozoa dramatically reduces the proportion of good-quality blastocysts on day 5.

Vanderzwalmen et al. [17] reported that the outcome of embryo development in a group of 25 patients after sibling oocyte injections with the four different grades of spermatozoa showed no differences in the fertilization rates as well in the rates of top-quality embryos on day 3. However, the occurrence of blastocysts formation was 56.3 and 61.4% with grades I and II spermatozoa, respectively (no significant difference), compared to 5.1% with grade III and 0% with grade IV, respectively ($p<0.001$).

In the same way, the rate of good-quality blastocysts was significantly reduced (2.9%) when all the oocytes in a group of eight patients were injected with grade III/IV spermatozoa, as compared to the use of only grade I/II spermatozoa (19.1%) ($p<0.01$) in a group of 34 patients.

Cassuto et al. [18] reported a statistically significant decrease in the fertilization rate according to the classes of injected spermatozoa (84% with class 1, 73% with class 2, 61% with class 3). In addition, a statistically significant relationship was noted when the comparison was made between the rate of expanded blastocysts (15% with class 1, 9% with class 2, 0% with class 3).

The size and the number of sperm nuclear vacuoles, identified accurately under the Nomarski optics, negatively affected blastocyst development and reinforced previous studies suggesting the early and late paternal effects on initial embryonic development [22–25]. The presence of a late paternal effect impacts embryo development after the onset of paternal DNA content contribution to embryonic development, which starts around day 3 after fertilization [25] and may often lead to early abortions.

These findings corroborate studies by Barth and Oko [26] and Thundathil et al. [27], who report that in the bovine species, nuclear vacuoles do not decrease fertilization rates but rather increase the incidence of early embryo losses.

Indications for IMSI

One of the most frequent questions with regard to IMSI relates to its indications.

History of Previous Failure of Implantation and/or DNA Fragmentation

As already mentioned in the previous chapter, MSOME-ICSI can be seen as a useful tool for patients with previous failure of implantation or for patients with high levels of DNA fragmentation [12, 14, 16]. Those previous studies were substantiated with recent data presented by Antinori et al. [28] and Nadalini et al. [29]. Antinori et al. [28] conducted a prospective randomized trial to assess the potential advantages of the IMSI technique (227 couples) over the conventional ICSI procedure (219 couples) in the management of patients with severe oligoasthenoteratozoospermia, regardless of the number of previous failed ICSI attempts. By comparing the two groups, the overall IMSI technique resulted in statistically significantly higher implantation and pregnancy rates than those for ICSI (17.3% vs. 11.3%, and 39.2% vs. 26.5%, respectively) in cases of severe male infertility, while no statistical differences were reported for miscarriage rates. Their data showed that the sub-group with at least two previous failed ICSI attempts received the greatest advantage from the IMSI procedure in terms of clinical pregnancy rate (29.9% vs. 12.9%). On the other hand, among these latter sub-groups of patients, no significant differences in miscarriage rates were reported, even though the clinical outcome was clearly in favour of the IMSI procedure, with a remarkable 50% reduction (17.4% vs. 37.5%).

Nadalini et al. [29] compared the reproductive outcome of couples with a history of at least one previous failed ICSI attempts who underwent IMSI with spermatozoa free of any nuclear morphological malformations (20 couples) with 37 couples referred for conventional ICSI treatment in the same period of time. As a result, the IMSI clinical outcome seemed to improve significantly in terms of clinical pregnancy rates (40% vs. 16.21%), and a positive trend towards reduction of miscarriages and biochemical pregnancies was seen.

Interestingly, the sub-group with fragmented DNA showed a significantly higher clinical pregnancy rate with respect to the ICSI control group (66.67% vs. 16.21%, $p=0.005$) [29]. They therefore suggested that sperm selection and oocyte microinjection based on high-power microscopy at >×6,000 appeared to significantly increase the pregnancy outcome of previous failed ICSI treatments, above all in patients with

several degrees of sperm DNA fragmentation corroborating the data of Hazout et al. [16].

Absence of Blastocysts in Previous IVF Cycles

We may also advice IMSI in case of absence of blastocyst formation after conventional ICSI treatment.

Unexplained Infertility

In case of unexplained infertility, a MSOME observation of the semen has to be advised. Our preliminary results in conjunction with those of Dr. Cassuto show a higher rate of class 4 [18] or grade IV [17] spermatozoa in an unexplained infertility patient as compared to a group of patients with pure tubal problem (personal observations).

General ICSI Candidates

Also, in our opinion, the question that brings a lot of debate is why not to select directly spermatozoa with the MSOME approach before ICSI?

The MSOME technique has to be considered as an additional tool for ICSI, particularly since the probability of selecting a normal spermatozoon is higher using the MSOME approach, as compared to the conventional ICSI approach.

Are we sure that with our conventional ICSI microscope, we can detect in all cases abnormal spermatozoa? Even after extensive search for patients who attend their first treatment, the frequency of selecting spermatozoa of normal morphological appearance greatly varies according to semen samples. We observed that in almost 50% of the semen sample, if selection had been performed using the classic ICSI approach, the likelihood of selecting sperm with a nuclear vacuoles would have been very high [17].

Considering the above, the following questions remain to be answered regarding the indications for IMSI: should IMSI be performed on all ICSI candidates or only on a sub-group of patients?

It is now obvious that assessment of sperm morphology by Kruger's strict criteria is routinely used and widely accepted as the best predictor of male fertility potential, better than sperm concentration or motility, highlighting the concept that sperm morphology is the most important parameter in the semen analysis. Everybody believes in this concept because the spermocytogram is one of the basic routine examinations.

As a consequence, we have to do all we can to select the best spermatozoa. There are absolutely no indications to select bad-quality spermatozoa if good ones are present in the prepared semen sample. Are there still indications where improved sperm selection before fertilization is not necessary or low-magnification microscopy using Hoffman modulation contrast is more than enough? Probably none.

At present, there is a big debate on which patients IMSI instead of ICSI should be recommended. Is it possible to find a threshold of normal spermatozoa above which selection at ×400 under Hoffman modulation contrast is sufficient?

There is a great heterogeneity between all the semen samples so that the frequency by which good spermatozoa can be selected varies greatly from one patient to the other. As consequence, the fundamental question to elucidate concerns the probability with which a normal spermatozoon can be selected with classical ICSI method under ×400 magnification instead of using the Nomarski optics.

The establishment of classification criteria, based on an assessment system, seems a valuable approach to determine a threshold limit for making the right therapeutic decision to advise ICSI or IMSI to patients entering an infertility treatment programme. For this reason, a prior screening of semen samples by MSOME to detect the presence of vacuoles would be a reasonable approach. But we have to recognize that at the present time, there are no studies reporting a threshold of percentages of normal forms for either recommending ICSI or IMSI.

Limiting Factors to Perform IMSI

However, there are some limiting factors that render MSOME selection almost not possible at all. In case of severe oligoteratozoospermia, the rare spermatozoa that are found are selected for injection. Even if the semen exhibit 100% abnormal morphology, it is difficult to select the best "second choice" from the sperm population.

MSOME for Routine Laboratory Semen Analysis

In order to assess the usefulness of the evaluation of sperm morphology by MSOME, two studies were undertaken in a first instance by Bartoov et al. [11] who estimated the correlation between MSOME and the WHO routine method [4] and more recently by Oliveira et al. [30] who compared the MSOME evaluation with the Tygerberg classification criteria [31].

Both works conclude that the MSOME criteria appear to be much more restrictive, presenting significantly lower sperm normalcy percentages for the semen samples in comparison to those found after routine analysis by WHO criteria and the Tygerberg classification. In addition, MSOME

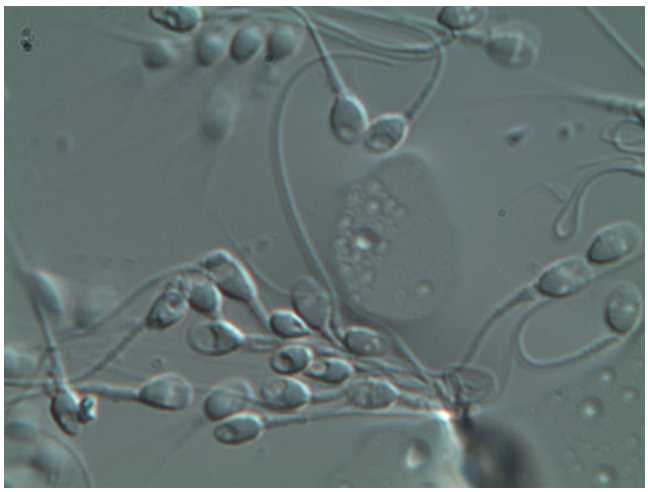

Fig. 26.1 Teratozoospermia semen observed by MSOME. DIC objective ×63 dry

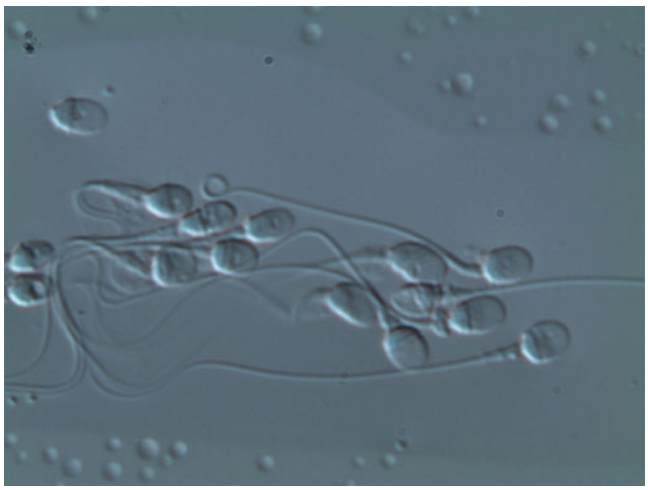

Fig. 26.2 Normal semen sample for conventional IVF observed by MSOME. DIC objective ×63 dry

represented a much stricter evaluation, since the use of Nomarski optics enabled the identification of vacuoles that could not be described with the same accuracy with the other methods.

These studies point towards a benefit in sperm morphology and quality evaluation by including MSOME among the criteria for routine laboratory semen analysis prior to ICSI or conventional IVF procedure. A previous MSOME spermocytogram revealing a high percentage of vacuoles may be judicious to propose directly IMSI as the best therapy for ICSI candidates (Figs. 26.1 and 26.2). Furthermore the additional information gained by MSOME may help to avoid fertilization failure in IVF cycles.

With the aim to define a predictive value of sperm normalcy using the MSOME on the outcome of combined IVF-ICSI, Wittemer et al. [32] undertook a study including 55 couples with previous failure of implantation after IUI treatments. In their next attempt, a combination of conventional IVF and ICSI was proposed for each couple. They conclude that below a threshold of 8% of morphological normal spermatozoa observed by MSOME, ICSI must be performed instead of conventional IVF in order to avoid the risk of fertilization failure.

The Importance of Selecting Morphologically Normal Sperm Without Vacuoles: A Phenomenon Raising a Lot of Questions

As described before, the main advantage of the MSOME technique is not only to select normal-shaped sperm but also to identify sperm which show special structural aberrations described as "vacuoles" in the sperm head. Although it seems to be well proven by the literature that the appearance of these structures is strongly related to reduced blastocyst outcome as well as pregnancy and implantation rates, we still do not know if there is a correlation between vacuoles and DNA damage.

Since it is more and more evident that vacuoles as well as DNA damage have a negative influence on the reproductive outcome, it would be valuable to comprehend if the presence of vacuole(s) in the sperm head is well correlated with DNA damage such as high degree of DNA fragmentation.

Effect of Vacuoles on Reproductive Outcome

In view of the evidence that nuclear defects negatively influence the outcome of embryo development, the argument that the oocyte itself supplies all the primary machinery for embryo development and that the sperm DNA only plays a secondary role probably has to be revised [33, 34]. Therefore, the pending question relates to the meaning of vacuoles, to their origin and, more importantly, to their possible negative effects they might have on the health of the offspring.

Effect of DNA Damage and Chromatin Disorganization on Reproductive Outcome

DNA damage in spermatozoa can affect both mitochondrial as well as nuclear DNA and can be induced by several mechanisms during the production of the spermatozoa (abortive apoptosis during meiosis, faulty chromatin remodelling during spermiogenesis) and/or mainly by oxidative stress during sperm transport through the seminiferous tubules and the epididymis or by endogenous caspases and endonucleases. Exogenous factors such as damage induced by radiotherapy and chemotherapy and by environmental toxicants can also induce DNA damage.

Several reports [35, 36] have shown that DNA damage is higher in the caudal epididymal and ejaculated sperm compared with testicular spermatozoa reinforcing the idea that during transport through the epididymis, levels of DNA fragmentation are higher.

We may consider DNA damage in three different forms: (1) fragmentation of the DNA in the form of single-stranded or double-stranded DNA strand breaks, (2) nuclear protein defects that may interfere with histone to protamine conversion and subsequent DNA compaction and (3) chromatin structural abnormalities causing altered tertiary chromatin configuration [36].

Such abnormalities of the paternal genome may affect blastocyst development [37–39] and pregnancy outcome [40, 41] after ICSI [42].

According to Hammadeh et al. [43], the integrity of the sperm chromatin may play the most important role, particularly in ICSI, in which most of the natural selection mechanisms are bypassed. In recent reports, Aitken [44] and Barratt et al. [45] report the possible negative influences of sperm DNA fragmentation both on pregnancy outcome and even on the next generation.

The argument that the oocyte supplies all the primary material (proteins and RNAs) and that the spermatozoa and their DNA only play a secondary role needs probably revision [33, 34]. A spermatozoon contains almost 3,000 different kinds of mRNA, some of which contain the code for proteins needed for early embryo development.

Is There a Correlation Between DNA Damage and the Presence of Vacuoles?

According to Berkovitz et al. [13], Cayli et al. [46] and Hazout et al. [16], vacuoles may reflect molecular defects responsible for anomalies of sperm chromatin packaging and abnormal chromatin remodelling during sperm maturation which, in turn, may render spermatozoa more vulnerable to DNA damage. According to Gopalkrishnan et al. [47], Hammadeh et al. [43] and Virro et al. [37], 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.

In order to substantiate these notions, recent studies [48–51] evaluated the integrity of the chromatin and the DNA fragmentation using the acridine orange staining procedure, aniline blue and the TUNNEL technique. All studies concluded that there is an obvious association between large vacuoles and high levels of denatured DNA. Garolla et al. [49] observed significantly better mitochondrial function, chromatin status and less aneuploidies in spermatozoa when nuclear vacuoles were lacking. For Franco et al. [48], there is an obvious association between large vacuoles and secondary as well as tertiary DNA structure damages in sperm nuclei: the association between high levels of denatured DNA in spermatozoa with large nuclear vacuoles suggests premature decondensation and desegregation of sperm chromatin fibres. The chromatin material of spermatozoa from patients with early pregnancy loss seems to be often either compact or partially compact and to have irregular nuclear borders with larger vacuoles. A regression analysis of 538 semen samples demonstrated that percentages of normal nuclear sperm and all spermatozoa with abnormalities of nuclear form at high magnification negatively correlated with percentages of DNA fragmentation. On the other hand, there was a positive correlation between percentages of spermatozoa with nuclear vacuoles and those with DNA fragmentation. Oliveira et al. [51] showed that both normal and abnormal nuclear forms appear to be equally vulnerable to DNA fragmentation as analysed under high magnification. The only sperm type that correlates with a high rate of DNA fragmentation is the category of sperm with >50% vacuolated nuclei, showing that selecting a spermatozoon based on morphology or motility is not a good criterion of DNA integrity.

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 vacuoles which are accurately evaluated at high magnification by MSOME [30], 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.

When and Why Vacuoles Are Formed?

After analysing the influence of vacuoles on embryo development, another question to investigate concerns their origin.

A study by Peer et al. [52] demonstrated that after 2 h of incubation at 37°C in culture media, the incidence of spermatozoa with vacuolated nuclei was significantly higher compared to incubation at 21°C. They suggest that prolonged (≥2 h) sperm manipulations for assisted reproduction therapy should be performed at 21°C rather than 37°C.

Another hypothesis proposed by Kacem et al. [53] states that sperm nuclear vacuoles, as assessed by MSOME, are mostly associated with the presence of acrosomal material. Their data suggest that the vacuole-free spermatozoa that are microinjected during IMSI are mostly acrosome-reacted spermatozoa free of acrosomal enzymes, such as trypsin-like acrosin that may induce a harmful effect [54].

Several experiments were conducted in our centres in order to analyse if we could perceive the formation of vacuole in real time. We conducted a 24-h time-lapse recording approach, first on selected grade 1 spermatozoa incubated

for 24 h at 37°C in culture media and in media containing different inducers of ROS (media in contact for 48 h with pool of plasma seminal containing high level of leucocytes or in media supplemented with different concentrations of H_2O_2). As compared to the control group, no changes in the morphology of the spermatozoa were observed. No vacuoles appeared. Even when the same experiment was conducted on spermatozoa with vacuoles, no changes in the size and shape of the spermatozoa after 24-h incubation at 37°C were observed (personal observation).

The same experiment was conducted with acrosome inducer (ionophore), but no change could be noticed. It is not fully understood at which time point in spermatogenesis vacuoles are formed and why they are such important indicators of sperm quality.

However, vacuoles seem to appear during the last maturation step of round spermatids. Could vacuoles be a selective mechanism of sperm to be removed in the natural selection process? We know that in sperm "incomplete apoptosis" is a common phenomenon [55]. Spermatozoa which do not pass the "quality control" due to, e.g. DNA defects or other aberration during spermatogenesis undergo the normal pathway towards apoptosis, but are not removed by phagocytes. Maybe the formation of vacuoles is a mechanism for abnormal spermatozoa to be attacked by ROS during storage and thereby being discarded.

Blastocyst Formation and Pregnancies with Vacuolated Spermatozoa: How Come?

Even though a negative impact of vacuoles is recognized, we still observe in some cases the formation of blastocysts followed by an ongoing pregnancy after IMSI with spermatozoa showing nuclear defects. How can we explain the ability of embryos to develop to the blastocysts stage and give rise to pregnancies after oocyte injection with spermatozoa exhibiting several small and/or large vacuoles?

It is no doubt that vacuoles have to be considered with attention. Several explanations such as the type and morphology of vacuoles, their location, the level of oxidative stress and repairing factors are still in suspend and may contribute in various ways or not at all to embryo development.

Type of Vacuoles

Spermatozoa contain heterogeneous vacuoles varying in number, size and content. To simplify this matter, we only refer to the term vacuole in any circumstance. But is vacuole the appropriate terminology? In a biological point of view, vacuoles are vesicles surrounded by a membrane. In the case of sperm vacuoles, what we call vacuoles represent more of a kind of depression in the nuclear part of the head. Some are deep, like a crater, or hollow. Watanabe et al. [56] reports that vacuoles are actually hollow whereby the plasma membrane falls into the nucleus, and Westbrook et al. [57] even speaks about nuclear crater formation in the sperm head. Toshimori [58] differentiates between large vacuoles with amorphous substances or membranous structures inside and small vacuolous patterns without any structures inside.

So the definition on its own is still an issue of controversial opinions and may explain why some spermatozoa can produce blastocysts and offsprings.

Location of Vacuoles

In a recent study, Hammoud et al. [59] analysed the rate of DNA fragmentation according to the position of the vacuoles on the sperm head. They report statistically higher degree of fragmentation if vacuoles are located in the posterior region. So it is also important that independently of the classification system, the location of the vacuole is taken into account.

Level of Endogenous and Exogenous ROS

The presence of vacuoles may reflect molecular defects responsible for abnormal chromatin remodelling during sperm maturation and might render sperm cells more vulnerable to DNA damage [46]. 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.

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 [60, 61]. We may then postulate the possibility to obtain blastocysts that will further develop to 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 towards DNA decays [60], especially if young and/or good-quality oocytes are capable of repairing and rescuing the DNA of poor-quality spermatozoa. As 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 foetal development.

However, we cannot determine whether the oocyte would be capable of repairing this damage. This mainly depends on the degree and the type of sperm DNA damage (single-stranded vs. double-stranded damage) [62]. Also, oocytes whose DNA repair mechanisms are not functional (ageing oocytes—in vitro culture conditions) or that have been damaged by endogenous (e.g. free radicals) or exogenous (e.g. radiation, environmental toxicants) factors might not be able to repair this damage.

Long-Term Safety

There remain concerns about the long-term safety of injecting spermatozoa carrying vacuoles. It is already well accepted that spermatozoa from older and/or infertile men have a higher level of imprinting disorders [62, 63]. The approach of selecting normal-appearing spermatozoa helps the oocytes in the DNA repair operations. However, when the DNA repair capacity in the oocyte is over passed, incomplete or mismatched, DNA repair mechanisms may lead to mutations. Under these circumstances, the damage may either remain unrepaired or be aberrantly repaired, creating DNA mutations. We have to be cautious, especially in the light of Aitken's work [44] on the possible negative effects of sperm DNA fragmentation in the next generation. Depending on the level of sperm nuclear DNA fragmentation, oocytes may partially repair fragmented DNA, producing blastocysts able to implant and produce live offspring. However, the incomplete repair may lead to long-term pathologies. The data of Fernández-Gonzalez et al. [64] in the mice model indicate that the use of DNA-fragmented spermatozoa in ICSI can generate effects that only emerge in later life, such as aberrant growth, premature ageing, abnormal behaviour and tumours from the mesenchymal lineage.

Up to now, there have not been sufficient numbers 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 damaging for the embryo and offspring [65]. According to a recent paper [66], sperm nucleus morphological normalcy, assessed at high magnification, could decrease the prevalence of major foetal malformations in ICSI children.

Therefore, how should we treat patients carrying 100% large vacuoles in their sperm samples? If such observation correlates with a positive DNA fragmentation test, antioxidant therapy may be proposed several months before the IVF treatment. But at the present, none of the studies report a significant benefit of specific antioxidant molecules therapy.

Knowing the potential risk of injecting abnormal spermatozoa, shall we continue the attempt with the patient's sperm or propose a testicular biopsy before proposing the option of a sperm donor if possible? As previously stated, reports have shown that sperm DNA damage is significantly lower in the seminiferous tubules compared with the cauda epididymis [67, 68] or ejaculated sperm [35]. Moreover, the use of testicular sperm in couples with repeated pregnancy failure in ART and high sperm DNA fragmentation in semen resulted in a significant increase in pregnancy rates in these couples.

Technical Aspect

For routine application and also to render the technique more user friendly, observations at ×1,000 magnification might suffice to select the right spermatozoa.

Some are reluctant to apply this new approach of selecting spermatozoa before ICSI. It is generally advocated that besides being time-consuming, IMSI is also expensive. The minimal requirement to observe and select spermatozoa using the MSOME criteria in a routine way consists to place the sperm suspension in a glass bottom dish and perform the observation under an inverted microscope equipped with Nomarski differential interference contrast optics with 63 and ×100 objective.

The adjunct of a vario-zoom, the amplification of the microscopic image with high-definition digital video camera, the video monitor as well as the computer software are not a priority to perform the observation and selection in a first instance.

Observation of moving spermatozoa at high magnification on the screen is difficult due to a more restricted field of observation.

The use of high magnification (>×6,000) in conjunction with digital image capture system permits to analyse spermatozoa in details. For example, MSOME spermocytogram can be done after capturing and storing several pictures of the prepared semen sample for later sperm morphological analysing.

Even though intracytoplasmic microinjection based on MSOME is a more time-consuming procedure, this should not be an argument not to start to perform IMSI. In order not to induce adverse affects to the oocyte quality, the IMSI procedure has to be envisaged in a way that the oocytes stay for a short time out of the incubator.

For both procedures, all the manipulations (observation, selection and injection) are done in glass bottom dishes. The first option consists to select the spermatozoa using the Nomarski microscope and perform the oocyte injection on a classical ICSI microscope.

A suspension of prepared spermatozoa is deposited in a drop of PVP. Morphologically motile spermatozoa are selected with an ICSI pipette and placed in a small drop of culture media. After collecting spermatozoa—if possible, 1.5× the amount of oocyte to inject—the dish is placed back

in the incubator for temperature recovery. After this period of incubation (~15 to 30 min), the oocytes are placed in HEPES culture media, and ICSI is performed on a conventional Hoffman microscope. The advantage of this approach is that the IMSI microscope is not occupied for a too long period and that the oocytes finally stay out of the incubator only for the injection step.

When the purpose of the study is to follow the spermatozoa individually at very high magnification (observation on the screen at >×6,000 magnification), a maximum of two oocytes are placed in the dish. With this technique, a minimum of two dishes are prepared. This technique is easy to perform if dry objectives are used so that we have no problem with oil when we change the dish.

Conclusions

IMSI is now a reality in ART practice but still with a lot of question marks regarding (1) the terminology of vacuoles, their classification, their location on the sperm head, and their origin and meaning, (2) the application of IMSI instead of IVF in cases of unexplained infertility, (3) the age of the woman; is IMSI necessary in younger women with good-quality oocytes where cytoplasmic factors might be able to repair sperm nuclear defects? and (4) the technical aspect. We have to be aware that this technique is demanding and has to be performed in the best working conditions not to impair the oocyte quality.

The introduction of IMSI has the advantage that embryologists realize that more attention has to be paid during sperm selection even in case of classical ICSI. The application of IMSI leads to more and better quality blastocysts and, as consequence, it increases the chance to select the proper embryo for transfer with high implantation potential. Even though there is no real proof in the human species on the abnormal outcome generated by spermatozoa carrying vacuoles, a higher and better-resolution technique has to be added as an additional tool for ICSI knowing the possible consequence of sperm DNA damage for offsprings.

Furthermore, the effect of using MSOME approach to select spermatozoa manifests itself when it is performed in combination with day 5 embryo culture of all fertilized oocytes.

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27

Sperm Testing and ICSI Selection by Hyaluronic Acid Binding: The Hyaluronic Acid-Coated Glass Slide and Petri Dish in the Andrology and IVF Laboratories

Gabor Huszar

Abstract

It is well established that the testis-expressed HspA2 chaperone is a measure of human sperm cellular maturity and function, including fertilizing potential. The presence of HspA2 in the synaptonemal complex also provides the link between low HspA2 expression and increased frequencies of chromosomal aneuploidies in arrested maturity, dysmature sperm. There is also a relationship between the levels of HspA2 expression in elongated spermatids and the related spermiogenetic events, such as cytoplasmic extrusion, formation of the normal sperm shape, the nuclear events of histone-transition protein-protamine replacement, and the associated changes in DNA packing, as well as the sperm plasma membrane remodeling. The membrane remodeling facilitates the formation of the zona pellucida (and the hyaluronic acid [HA]) binding sites, which enables the sperm to fertilize and provide paternal contribution to the embryo. In dysmature sperm, some or all of these developmental steps are arrested (there is a well-established relationship between nuclear and cytoplasmic dysmaturity). For this reason, the sperm selected by the zona pellucida or by hyaluronic acid are comparable via the common origin of plasma membrane remodeling and the formation of both receptors. With the advent of ICSI, the challenge to understand which sperm is empowered to fertilize the egg and the ongoing research focusing on biochemical markers of sperm function have taken higher prominence, as the pathology in male infertility patients, who require ICSI treatment, of higher complexity. The HA receptor of mature sperm, coupled with HA-coated slides or Petri dishes, allows the direct visual observation of sperm-HA binding, which is the basis for sperm maturity testing, and the assessment of sperm binding to HA which is related to sperm-zona pellucida binding. Sperm-Ha binding provides a major advancement in semen evaluation and it also facilitates; the selection of single mature sperm for ICSI [3]. The HA-binding step, similar to zona pellucida binding, eliminates dysmature sperm that exhibit cytoplasmic retention, persistent histones, and DNA chain breaks. Further, the frequencies of sperm with chromosomal disomy and diploidy are reduced by 4–6-fold in HA-bound sperm vs. semen sperm fractions [3]. This reduction is similar to the increase of chromosomal aberrations in ICSI children. Combined studies of sperm shape and chromosome probes demonstrated that sperm shape does not reliably aid selection of haploid sperm [34]. Thus, HA-mediated sperm selection is a novel and efficient technique that may alleviate potential future public health problems that are related to ICSI with visually selected sperm, such as chromosomal aneuploidies, shortened lifetime, and increased cancer rates.

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Biochemical markers of sperm maturity • Hyaluronic acid binding • Andrology evaluation/ICSI selection of sperm • DNA integrity and chromosomal aneuploidies

Overview of Sperm Biomarkers

Infertility has been commonly defined as failure to conceive following 12 months of regular intercourse. In the 2008 national US statistics, 35% of the IVF-ET cycles reported in the United States were diagnosed with male factor infertility either as a single (17%) or combined (18%) diagnosis.

Semen analysis is routinely used to evaluate the male partner of the infertile couple. Although widely used normal semen measures have been published by the WHO, the current normal parameters for sperm concentration, motility, and morphology fail to meet rigorous clinical, technical, and statistical standards [1]. With the advent of ICSI [2], it is imperative to provide solid scientific base for the characterization of sperm that are enabled to fertilize and provide paternal contribution. The ongoing research, focusing on biochemical markers of sperm function [3], have taken high prominence, as the pathology in male factor infertility patients, who require ICSI treatment, is likely to be of high complexity.

In the past 2 decades, the Huszar laboratory made major advances upon the biochemical markers of human sperm maturity and function [4–6]. In this chapter, an overview is presented, including clinical application of the biochemical markers, the cell biology of sperm maturity and genetic integrity, the lack of relationship between sperm shape and chromosomal aneuploidies, sperm hyaluronic acid (HA) binding, the application of sperm-HA binding score in the Andrology laboratory, and preferential selection for ICSI spermatozoa that fully completed cellular development.

In the early phase of sperm studies, we were searching for an objective biochemical parameter that may predict sperm fertility, independently from the classical semen parameters. In assessments of B-type creatine kinase (CK), we found significantly higher sperm CK content in men with diminished fertility [4, 7, 8]. One of the first recognized markers was cytoplasmic retention which was measured by the excess cytoplasm in sperm; biochemically this observation translated to an increased sperm creatine phosphokinase (CK) activity [8]. The variations of CK activity in semen samples have been well documented, as well as the fact that sperm recovered from pellets of density gradient centrifugation had substantially lower CK content [9]. A logistic regression analysis of 180 couples with oligozoospermic husbands, treated with intrauterine insemination (IUI), indicated that the increased levels of CK activity in these oligozoospermic, men predicted a lower likelihood for pregnancy [8, 10]. The CK-immunostaining patterns indicated that the high sperm CK activity was a direct consequence of increased cytoplasmic protein and CK concentrations in individual spermatozoa [11]. This suggested a sperm developmental defect in terminal spermiogenesis when the surplus cytoplasm is normally extruded from elongating spermatid [12, 13].

We have also found another sperm protein with ATP content in mature sperm [14]. This protein was later characterized as the 70-kDa testis-expressed HspA2 chaperone [15]. Thus, the proportional concentrations of CK and HspA2 (or HspA2 ratio) reflect the representation of fully developed and dysmature spermatozoa in semen samples [14]. In three studies (approximately 500 men), there were similarly close correlations between sperm CK activities and HspA2 ratios ($r = -0.69$, -0.71, and -0.76; $p < 0.001$; $N = 159$, 134, and 194) [14, 16, 17]. The proportion of mature and immature sperm, whether the men were normozoospermic or oligozoospermic, showed a day-to-day and man-to-man variation in semen samples [15]. Indeed, in a study of couples treated with IUI, a logistic regression analysis indicated that sperm CK activity did, but sperm concentrations did not, provide a predictive attribute for pregnancies to occur [10].

In further studies of sperm-hemizona (halved unfertilized human oocytes) complexes, all zona pellucida-bound sperm were clear-headed without cytoplasmic retention. Thus, immature sperm with retained cytoplasm and low expression of HspA2 were deficient in the zona-binding site [9]. We hypothesized that during normal spermiogenesis, a plasma membrane remodeling step occurs. In a blinded study, focusing upon β1, 2,-galactosyltransferase (present exclusively in the sperm plasma membrane), the cytoplasmic content of CK or HspA2, and the membrane density of β1-, 2-galactosyltransferase were closely related (both $r > -0.8$ and > 0.8, respectively, with $p < 0.001$) [18]. Thus, during normal spermiogenesis and plasma membrane remodeling, along with formation of the zona pellucida-binding site, and as found later, the formation of the hyaluronic acid (HA)-binding site also occurs, which is the basis for the HA-mediated fertility testing and ICSI sperm selection in men.

Male Fertility and Semen Parameters

Contemporary studies, directed to the relationship between semen attributes and male infertility, focus on the experience with IUI which, from the perspective of sperm-zona pellucida interaction, is similar to that of natural conception. There are

two major IUI studies on the relationship between semen parameters and fertility. One is a major collaborative effort conducted at seven sites utilizing approximately 1,600 couples [19]. The other work summarizes the data of 26 IUI publications encompassing over 30,000 cycles in 14,000 couples [20]. The authors of both studies concluded that assessment of single sperm attributes, even strict sperm morphology, is of limited predictive value. These data confirm our findings regarding the predictive value of biochemical markers with no contributions by sperm concentrations or motility [10].

With respect to IVF-ICSI data, several investigators attempted both IVF and ICSI in the same cycle on sibling oocytes [21–24]. In the van der Westerlaken study using 106 couples (1,518 oocytes), 28 of the couples were treated with ICSI, while 78 couples were treated with both IVF and ICSI. Two couples failed to fertilize in both IVF and ICSI, while the 26 women who were treated only with ICSI showed a 57% fertilization rate (182 oocytes). In the remaining 78 patients, 528 oocytes were treated with conventional IVF yielding a 51% fertilization rate, and 858 oocytes were treated with ICSI resulting in a 56% fertilization rate. The pregnancy rates similarly fail to show differences, as the transfer rates were 54% for IVF and 48% for ICSI.

It seems that above a threshold motile sperm density, no semen parameters are identified that would predict whether IVF or ICSI is more beneficial toward for a particular couple. The data of sperm-HA binding and ICSI with HA-selected sperm support that the sperm-HA binding assay contributes to an objective assessment of sperm function and male fertility and thus optimize the mode of fertility treatment. ICSI selected with hyaluronic acid binding (the PICSI dish) is increasingly efficient in the below 60%, low sperm-HA binding score level (high incidence of dysmature sperm). The ability to measure the proportion of sperm that are able to bind to the zona pellucida greatly enhances the benefits of semen analysis. In addition, considering sperm concentration, motility, and morphology, our understanding regarding the pathogenesis of male infertility is improved. Indeed, the sperm-HA binding assay is more on target compared to the so far most advanced approach, the sperm-hemizona assay, as the HA slides are of uniform quality as opposed to the functional variations among unfertilized human oocytes.

Spermiogenetic Events and Sperm Fertilizing Function

From the perspective of male gamete evolvement. It is important that synthesis of the HSP70 family of chaperone proteins (HspA2 in men) is regulated developmentally and that they contribute to the maintenance of the synaptonemal complex and to the meiotic function during spermatocyte development [25–28]. With respect to human sperm, our laboratory was the first to demonstrate the two-wave expression of HspA2 (Fig. 27.1), first in spermatocytes as a meiotic component within the synaptonemal complex and second in elongating spermatids during terminal spermiogenesis [15]. Further, in human and stallion studies, we found that all CK and HspA2-related maturational events are completed in sperm cells prior to reaching the caput epididymis [29].

In considering the potential diagnostic value of the sperm-HA binding assay, it should be noted that the relationship between male fertility and the conventional semen

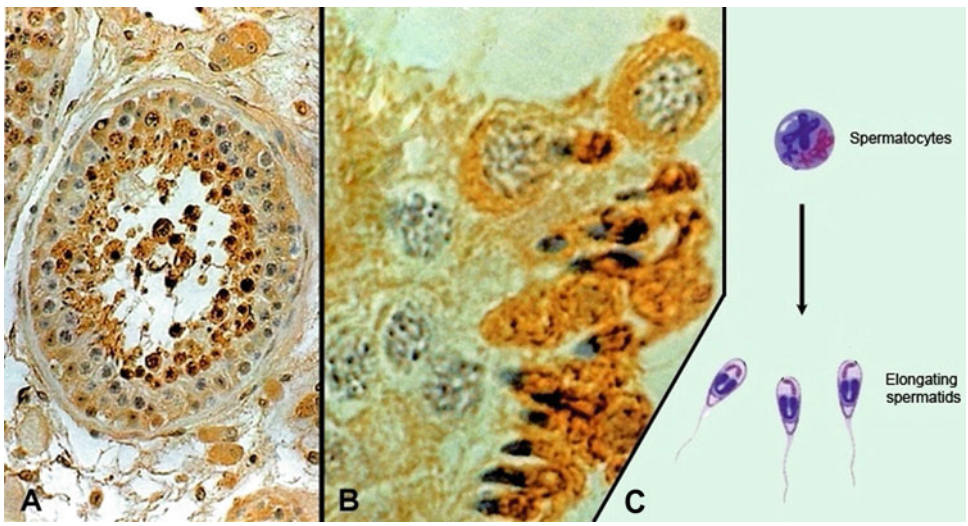

Fig. 27.1 Human testicular biopsy tissue immunostained with heat shock protein HspA2 antiserum. (**a**) Illustrates the structure of the seminiferous tubuli and the staining pattern of the adluminal area. HspA2 expression begins in meiotic spermatocytes, and there is a second wave of expression in elongating spermatids (**b**, **c**) (from Huszar et al. [3], with permission)

parameters is inconsistent. In addition to the clinical utility of sperm CK activity [10], expression levels of the sperm HspA2 chaperone protein were also tested in blinded studies of in vitro fertilization couples. In one blinded study, we assigned 84 husbands from the Jones Institute and Yale, based only on their sperm CK-HspA2 levels (while their semen parameters or reproductive histories were unknown), into "high likelihood" (>10% HspA2) and "low likelihood" (<10% HspA2) for fertility subgroups [16]. All pregnancies were found in the "high-likelihood" group. An additional benefit of the HspA2 levels was apparent: 9 of the 22 "low-likelihood" men were normozoospermic but had sperm dysmaturity. Thus, the HspA2 level provides a diagnostic utility for unexplained male infertility (infertile men with normal semen [16]). Recently, the utility of HspA2 levels was reexamined in 194 couples treated with IVF at Yale. The ROC analysis showed a 100% predictive value for failure to achieve pregnancy in the range of <10.4% HspA2 threshold. As in the 1992 study, 9 of the 15 men with <10% HspA2 ratio (18 of the 37 men (45%) in the two studies) and pregnancy failure were normozoospermic; thus, their diminished fertility would be unsuspected [30]. In summary, the expression levels of HspA2 correlate well with sperm cellular development and IVF success.

Sperm Structure and Function: Nuclear and Cytoplasmic Biochemical Markers

The recognition that HspA2 reflects the meiotic process, as well as the cytoplasmic and nuclear downstream events of cellular development in spermatozoa, was a key advance [3, 15, 112]. The spermatid phase is a dividing point between normally developing and diminished maturity sperm. In elongating spermatids with a substantial upregulation of HspA2, *normal sperm development* occurs with the orderly extrusion of cytoplasm and simultaneous plasma membrane remodeling, yielding a spermatozoon with normal head morphology and fully formed binding sites for the zona pellucida and hyaluronic acid (Fig. 27.2).

In contrast, spermatids with low HspA2 expression (sperm dysmaturity) show increased cytoplasmic retention and higher frequencies of chromosomal aneuploidies due to synaptonemal complex defects, as well as DNA chain breaks as a result of inadequate delivery of DNA repair enzymes. The latter is likely due to diminished HspA2 chaperone activity [25, 26]. These dysmature sperm are characterized by cytoplasmic retention, abnormal head morphology, increased levels of reactive oxygen species, and consequential DNA

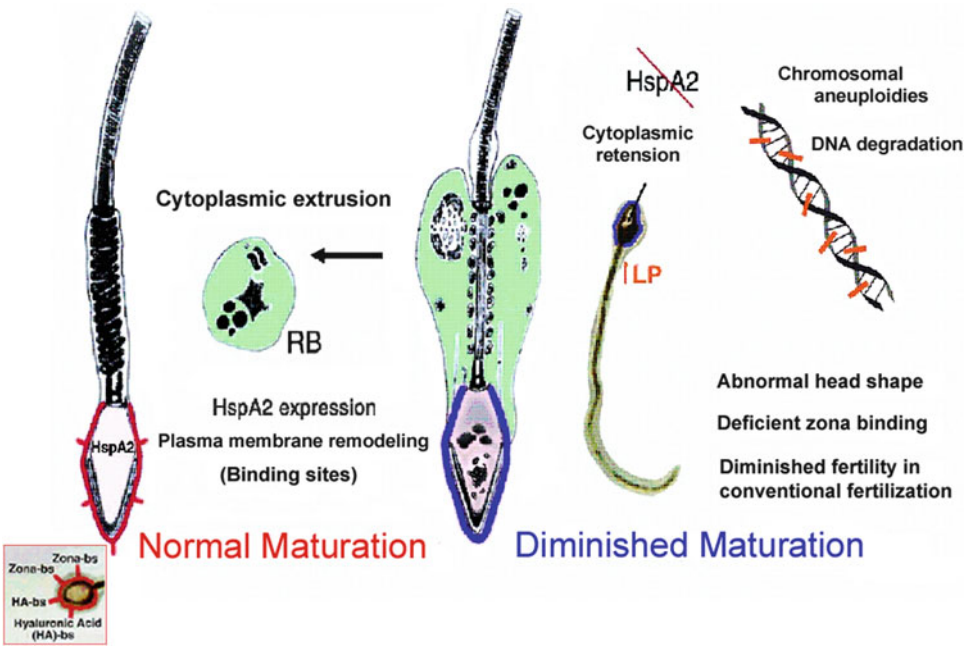

Fig. 27.2 A model of normal development and dysmaturation of human spermatozoa. In normal sperm maturation (*left*), elongating spermatids undergo cytoplasmic extrusion (represented by the loss of the residual body, RB) and plasma membrane remodeling, leading to the formation of the zona pellucida and hyaluronic acid-binding sites (bs) (change from *blue* membrane to *red* membrane with the stubs). Dysmature spermatozoa have low heat shock protein HspA2 expression, which may causes meiotic defects and chromosomal aneuploidies. Also, in dysmature sperm, there is a higher retention of CK and other cytoplasmic enzymes, increased levels of lipid peroxidation (LP) and consequential DNA fragmentation, abnormal sperm morphology, and deficiency in the zona and hyaluronic acid-binding sites (from Huszar et al. [3], with permission)

Fig. 27.3 Zona pellucida binding of normally developed and dysmature human spermatozoa. *Left*: Normally developed and dysmature sperm (cytoplasmic retention and abnormal morphology are highlighted with creatine kinase immunostaining) in dysmature spermatozoa. Also, the plasma membrane remodeling is illustrated by the *red* and *blue* colors, respectively. *Right*: Sperm-hemizona complexes immunostained with creatine kinase antiserum. Please note that only the clear sperm without cytoplasmic retention and normal morphology are able to bind to the zona pellucida (from Huszar et al. [46], with permission)

fragmentation. Further, due to the incomplete spermiogenetic process, the plasma membrane remodeling and the formation of the zona pellucida and hyaluronic acid-binding sites fail to occur. Thus, dysmature sperm are unable to bind to the zona pellucida or fertilize via natural or IVF conception, only by ICSI. Conversely, none of the hemizona-bound spermatozoa have cytoplasmic retention (Fig. 27.3). The likely reason why such sperm do not demise prior to ejaculation is the presence of antiapoptotic protein Bclx2 in the surviving germ cells [9, 31].

Sperm Head Shape and Sperm Genetic Integrity: Shape or Dimensional Properties Do Not Facilitate the Selection of Haploid Sperm for ICSI

The potential relationship between abnormal sperm morphology and chromosomal aberrations has been of long-term interest [32, 33]. Earlier data supporting this association were based on the frequencies of abnormally shaped or aneuploid sperm in semen samples, but not within the same sperm. Examination of the same individual sperm for both shape and aneuploidies has become possible when the data from the Huszar laboratory established that sperm preserve their shape after undergoing the decondensation and denaturation steps that are a prerequisite of FISH analysis [34]. Based on this finding, we studied the potential role of shape attributes in ICSI sperm selection [35].

First, using objective shape measurements, by the Metamorph computer-assisted program, 1,286 individual sperm were evaluated from 15 men: 900 haploid cells, utilizing three-color FISH for chromosomes 17, X, and Y and two-color FISH for the 10 and 11 chromosomes, and all the disomic and diploid sperm images that were found in the samples (368 cells). Second, we sorted the 900 nonaneuploid sperm and classified into three groups as "small head," "intermediate head," and "large head." Third, we sorted the 256 aneuploid and 130 diploid sperm according to the head-size parameter ranges established in the nonaneuploid sperm group and determined the frequencies of disomies and diploidies within the "small," "intermediate," and "large" groups.

Aneuploidies and diploidies were present within all three categories. The proportions of the 256 disomic sperm in the small, intermediate, and large sperm head category groups were 66 (27 ± 2%), 56 (23 ± 1%), and 133 (50 ± 2%), respectively. Similarly, the mean number of diploidies in the three sperm head categories were 3.0 ± 1, 8.0 ± 1, and 89.0 ± 2%, respectively. Interestingly, approximately 27% of sperm with disomy and 3% with diploidy of the 386 nonhaploid sperm were among the 300 sperm were within the "small" and "normal" dimensions.

In another analysis of the 1,286 images, we examined sperm shape according to their characteristics as symmetrical ($N = 367$), asymmetrical ($N = 368$), irregular ($N = 504$), and amorphous ($N = 47$). Disomic and diploid sperm were present in all four groups with an increasing frequency of 18, 18, 41, and 98%, respectively [35]. Finally, according to the Kruger strict morphology method as normal and abnormal, the proportion of sperm with normal morphology in the symmetrical, asymmetrical, irregular, and amorphous groups was 26, 3, 1, and 0%, respectively. There were aneuploid sperm even within the Kruger normal group.

Thus, it is evident that visual shape assessment, i.e., choosing the "best-looking" sperm, is an unreliable method for ICSI selection of haploid spermatozoa [35, 36]. This inconsistent relationship between sperm chromosomal aberrations and sperm shape seems to be in contrast with the ICSI sperm selection approach based on enhanced microscopic imaging. However, there were no studies yet on sperm chromosomal aneuploidies,

persistent histones, or DNA chain integrity in sperm selected by the high magnification imaging approach [37, 38].

Testing of Sperm Development by Hyaluronic Acid-Binding in the Andrology and IVF Laboratories

Concurrently with the studies on sperm development we investigated the effects of hyaluronic acid (HA) or hyaluronan on human sperm function. HA-containing medium increased sperm velocity, retention of long-term motility and viability with an immediate increase in sperm tail cross-beat frequency, and sperm velocity upon HA exposure [39, 40]. When these sperm were exposed to media lacking HA, the motility and velocity properties returned to those of the control sperm. Thus, the HA effects appear to be receptor-mediated [41–43]. In line with this idea, dysmature sperm with cytoplasmic retention, and thus retarded plasma membrane remodeling, were not stimulated by HA [39, 40].

Fully developed spermatozoa may selectively bind to solid-state HA. Indeed, sperm bound to HA were oriented head first, as with sperm-zona pellucida binding. This finding is in agreement with the HA binding pattern in monkey sperm [41, 44]. Also, in line with previous hemizona binding experiments, sperm bound to HA exhibited a uniform shape conforming to normal cells of the Kruger classification which is based on the zona pellucida-bound spermatozoa [35, 45–47]. Further attributes of HA-bound mature spermatozoa indicated that HA-selected sperm are viable. Nonviable sperm do not bind and may be removed from the HA slide with gentle washing (Fig. 27.4). HA-bound sperm are also devoid of cytoplasmic retention, persistent histones, DNA fragmentation, and the apoptotic marker caspase 3 (Fig. 27.5). Indeed, in a recent study, we demonstrated that each spermatozoa bound to the HA spot of the sperm selection PICSI dish exhibits green fluorescence (Fig. 27.6) with the acridine orange reagent (represent the lack of DNA chain degradation in the HA-bound sperm fraction) [48]. These properties are very important because nuclear and cytoplasmic sperm dysmaturity, particularly the presence of DNA fragmentation, is known to adversely affect the paternal contribution of sperm to the zygote [4, 13, 31, 34, 49–54].

The comparable sperm selection properties of zona pellucida and HA were further confirmed in a collaborative study between the Huszar and Kruger laboratories. In 37 of the 63 samples with <14% Kruger normal (teratozoospermic) score, the HA-bound sperm vs. semen sperm fractions showed an approximately fourfold enrichment in Kruger normal spermatozoa. The likelihood ratio was 3.04× (95% confidence limits: 1.9–4.7× considering the three blinded investigators). This enrichment is comparable to the 3.9× improvement reported by Kruger in the zona pellucida bound vs. semen sperm [55–57]. Since sperm-zona pellucida binding is the penultimate fertilization step, sperm-HA binding is very important evidence.

Relationship Between Sperm Binding to Hemizonae and Hyaluronic Acid

We tested the validity of the idea that the HA-binding and zona-binding sites in sperm are commonly regulated via plasma membrane remodeling in a comparative study of sperm-hemizona binding and sperm-HA binding scores in aliquots of the same sperm sample in 60 men [18, 46, 52, 58].

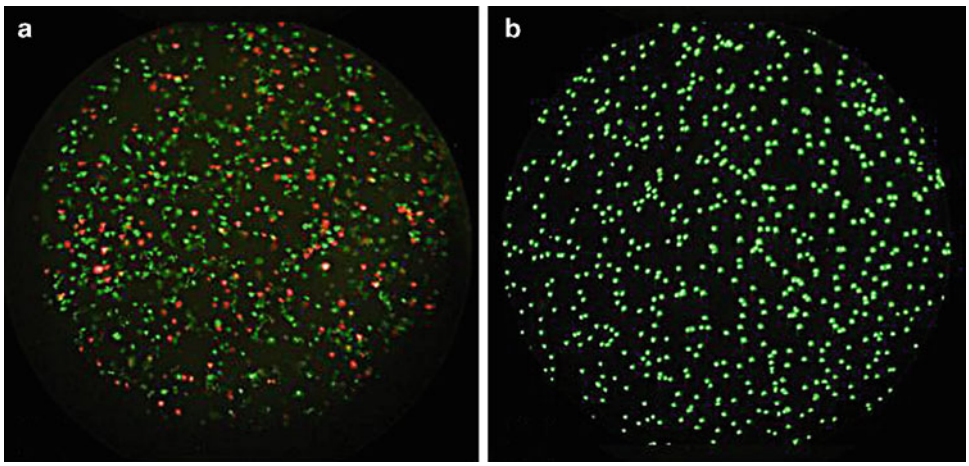

Fig. 27.4 Sperm viability and hyaluronic acid (HA) binding. (**a**) A drop of semen treated with a combination of Cyber green and propidium iodide, respectively, in order to highlight viable (*green*) and nonviable (*red*) spermatozoa. (**b**) HA-bound spermatozoa are gently rinsed and stained with the same combination of dyes. Only the viable green spermatozoa remain bound to the HA slide (from Huszar et al. [52], with permission)

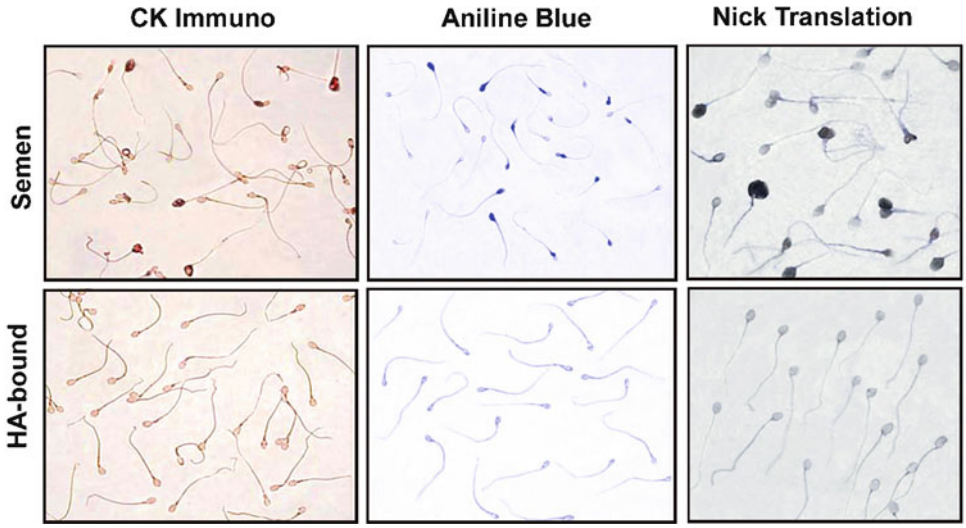

Fig. 27.5 Hyaluronic acid (HA)-bound mature spermatozoa do not contain cytoplasmic retention (creatine kinase [CK] immunostaining), persistent histones (aniline blue staining), or DNA fragmentation (detected by DNA-nick translation; Sati et al. [53]). *Upper*: Spermatozoa, including dysmature cells, from whole semen showing various degrees of staining. *Lower*: The HA-bound sperm stained with the respective cytoplasmic and nuclear biochemical markers. HA-bound normally developed spermatozoa show patterns clear from the biochemical markers of dysmaturity (cytoplasmic retention, persistent histones, DNA fragmentation)

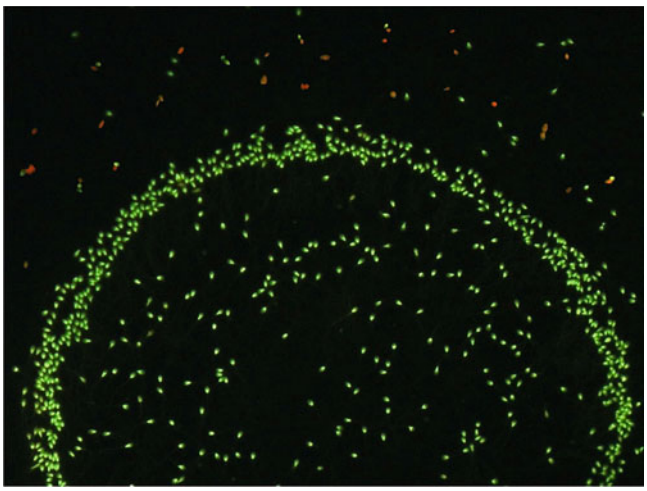

Fig. 27.6 PICSI dish picture. Acridine orange-stained sperm in the area of the HA-selection spot and in the outside control area of the HA spot-modified Petri dish (PICSI dish). Please note the almost exclusive presence of sperm with *green acridine orange* fluorescence (represent sperm with no DNA fragmentation) within the HA-selected sperm fraction. In the outside control area, the original semen is shown before HA selection with about 40% sperm with *red acridine orange* staining (sperm with fragmented DNA). The higher density of sperm around the perimeter of the HA spot is due to an HA ridge, which is caused by the application tool for placing the HA spot (48, with permission)

By using bisected unfertilized human oocytes and the HA-binding slides, there was a close correlation between the binding scores to either HA or hemizona ($r=0.73$, $p<0.001$, $N=54$) [59]. Thus, if the zona-binding properties of sperm were low or high, it was followed by a similar pattern by the HA-binding properties. Indeed, we suggest that the correlation is not closer because of the undefined sperm-binding ability of unfertilized oocytes, a factor which is more variable than the properties of the uniformly manufactured HA-coated binding slide.

A Semen Chamber Device for the Sperm-HA Binding Assay

Based on the fact that mature sperm selectively bind to solid-state HA, we have developed the sperm-HA binding assay, both as a clinical Andrology test and as a method for selection of mature sperm for ICSI. Sperm binding occurs within 8–10 min. The time-lapse photography highlights the pattern of motile sperm and arrested motile sperm (see examples in Fig. 27.7). The assessment of HA binding is based on the proportions of bound sperm with increased tail cross-beat frequency vs. the unbound swimming sperm that do not "perceive" the HA. Nonmotile sperm without tail movement are not considered [52]. In line with the inverse relationship between the spermiogenetic events of arrested cytoplasmic extrusion (increased CK retention), and sperm plasma membrane remodeling (formation of the HA-binding sites), there was a close correlation between sperm CK activity and HA binding ($r=-0.78$, $p<0.001$, $N=56$).

Because the clinical utility of sperm CK activity and HspA2 levels were already established in IUI and IVF studies [10, 16, 30], we formulated a general idea regarding the expected clinical application of the sperm-HA binding score. We considered three binding zones in 56 men: (1) ≥80% *binding* (excellent binding, $N=32$). In these men, the sperm

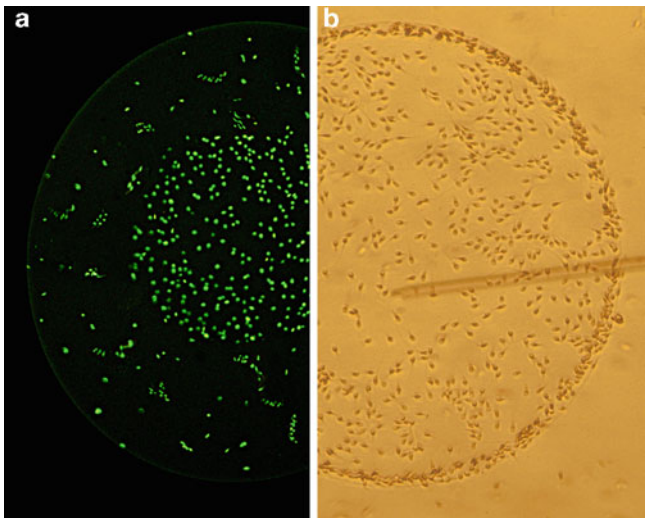

Fig. 27.7 Time-lapse photography of spermatozoa stained with Cyber green stain. *Left*: Semen drop placed on a glass slide. Please observe the progress of moving free spermatozoa. *Right*: A drop of the same semen observed on hyaluronic acid-coated slide. Most sperm (the normally developed ones) are bound by the sperm head (remodeled plasma membrane) to the hyaluronic acid and exhibit vigorous tail beating. Some dysmature sperm (no membrane remodeling) still swim freely and do not perceive the hyaluronic acid coating as they lack the hyaluronic acid receptors

CK activity was 0.18 ± 0.02 CK IU/10^8 sperm (normal range <0.25); thus, there was no male factor infertility or need for intervention. (2) *Binding between 60 and 80%* (intermediate binding, $N = 14$). In this group, the sperm CK activity was elevated to 0.50 ± 0.06 CK IU/10^8 sperm. These couples may benefit from IUI. (3) *Binding ≤60%* (diminished binding, $N = 10$). In this group, the CK activity was a high 2.8 ± 0.1 CK IU/10^8 sperm (tenfold higher than the normal sperm CK activity range), indicating a high level of sperm cells with cytoplasmic retention. These men should be retested, and if the diminished binding is confirmed, these couples may be best treated with ICSI after HA sperm selection. These application guidelines are being confirmed with prospective studies and clinical experience [86–88]. As with the other biochemical markers, HA binding was largely independent from sperm concentrations. Among men within the <20 million sperm/ml concentration range ($N = 18$ of 56 men), we identified three excellent, seven intermediate, and eight diminished HA binders [60].

Considerations in Men with Excessive Semen ROS Production

Sperm preparation for assisted reproduction in men with high seminal ROS may improve sperm quality if media containing antioxidants such as reduced glutathione or catalase/EDTA [61–63]. Such approach may improve the quality of gametes used by protecting the spermatozoa from high oxidative stress.

Another approach available is the hyaluronic acid-mediated sperm selection. The rationale for this approach is as follows: (a) A close correlation has been shown between ROS production and cytoplasmic retention which represent gamete dysmaturity [9]. Thus, it is expected that dysmature sperm, which produce and\or have been affected by ROS, would have a high amount of DNA degradation and due to the arrested spermiogenesis, would not bind to hyaluronic acid. (b) Sperm selection by HA binding is also helpful as hyaluronic acid-bound sperm is also devoid of excess persistent histones, DNA fragmentation, and the apoptotic process. Thus, sperm selection by deselection of dysmature spermatozoa, and positive selection of normally developed spermatozoa with low or no ROS production, is an appropriate and practical solution [3].

Another advancement is the utilization of sperm extracted from the testes (TESE sperm). It is established that sperm originating in the adluminal area are protected from oxidative attack, whereas most ROS-initiated DNA fragmentation occurs during epididymal storage [64]. A number of reports indicate that sperm DNA damage is significantly lower in the seminiferous tubules compared to the cauda epididymis or ejaculated sperm [64, 65]. The use of TESE sperm in couples with repeated pregnancy failure in ART, and high sperm DNA fragmentation in semen, resulted in a significant increase in pregnancy rates [66]. Thus, use of sperm with very low levels of sperm DNA fragmentation reduces the burden of sperm DNA repair by the zygote. With respect to sperm in the seminiferous tubuli and in the epididymis, the usefulness of the TESE approach is limited to the location of the testicular origin, i.e., whether sperm extracted from the testes would originate in the seminiferous tubuli, in which the spermatozoa was already released from the adluminal compartment after all aspects of the sperm cellular maturation has been completed [29]. No ICSI selection may be performed with the PICSI dish in TESE sperm, due to the lack of progressive motility in these testes-extracted spermatozoa.

The Key Role of Sperm Chromatin Maturation

The formation of fully developed spermatozoa is a unique process involving a series of steps in both the nuclear and cytoplasmic compartments including histone-transition protein-protamine replacement [67]. A greater than tenfold compaction of sperm DNA is achieved during the final phases of spermatogenesis when the normally occurring histone that is bound to DNA is almost completely replaced by protamines.

Earlier studies showed an association between male infertility and diminished histone-transition protein-protamine

exchange in sperm, and this may be detected by aniline blue staining of the excess persistent lysine-rich histones [68–72, 75]. Accordingly, based on the variations in sperm maturity, staining intensity was light, intermediate, and dark tone and represent sperm with mature, moderately immature, and severely immature developmental status, respectively [53]. It is clear that sperm chromatin is essential for sperm function and subsequent embryonic development because defects in sperm chromatin are linked to natural reproductive failures, such as spontaneous abortion as well as assisted reproductive failure [73–75].

The relationship between persistent histones and DNA degradation was highlighted in a recent report from the Huszar laboratory. In sperm with dark aniline blue staining, representing extensive levels of persistent histones, there was a lack of signal for fluorescence in situ hybridization (FISH) chromosome-probe binding [75]. Thus, deficiency in sperm chromatin development, improper DNA folding, and fragmented DNA chains caused diminished FISH probe binding, as there were limited number of long DNA sequences that facilitate the binding of FISH probes.

Regarding the interrelationship between sperm dysmaturity and persistent histones, a report by the Huszar group indicates that diminished sperm fertility and reduced paternal contribution of sperm to the embryo may not originate only in the arrested histone-protamine remodeling, but along with the persistent histones, there are other associated factors of dysmaturity in the same spermatozoa [53]. In these studies, a double-staining method was developed: First, sperm are stained with aniline blue for probing persistent histones. Second, after recording the aniline blue-stained sperm fields, and a subsequent destaining step, the same sperm are probed for cytoplasmic retention with creatine kinase immunostaining, for the apoptotic process with caspase 3 immunostaining, for DNA chain fragmentation with in situ DNA-nick translation, and for sperm shape [53].

The evaluation of the same sperm after the first and the subsequent probes convincingly showed an approximately 75% agreement between the aniline blue staining patterns whether light (no probe presence), intermediate (some probe detection), and dark staining (heavy probe presence) and of the various other biochemical probes, such as cytoplasmic retention, DNA degradation, caspase 3 apoptotic marker, or Kruger normal/abnormal sperm shape. This indicated that indeed, there is a relationship between the attributes of arrested development or dysmaturity within the same sperm. Also, the experiments demonstrated that the nuclear and cytoplasmic, as well as the early and late events of spermatogenesis and spermiogenesis, are related. Thus, sperm with persistent histones and arrested chromatin remodeling, and failing to initiate proper DNA folding in the nucleus, are also characterized by dysmaturity and diminished sperm function [53].

Does Sperm-HA Binding Test Predict High DNA Integrity: Studies with Two Different Approaches

In previous studies [4, 52] with the methods of in situ DNA-nick translation, it was demonstrated that HA-bound spermatozoa are viable; exhibit lack of cytoplasmic retention, persistent histones, and apoptotic probes; and also show high DNA chain integrity. Another recent paper, on acridine orange staining of HA-bound spermatozoa, a reagent which provides green fluorescence for DNA with high chain integrity and orange fluorescence for damaged DNA, Liu and Baker reported that the zona pellucida-bound sperm has mostly green fluorescence [76]. In the Huszar laboratory, the acridine orange assay was performed with sperm bound to the HA spot of the PICSI dish, a device which is used for ICSI sperm selection in IVF laboratories. The data indicated, using the very high quality Polysciences Inc. acridine orange, that literally, 100% of the hundreds of HA-bound sperm were of green fluorescence (Fig. 27.7). Thus, whether we probe DNA integrity with nick translation or acridine orange reagent, the DNA of HA-bound sperm had high integrity [3, 48]. This experiment using the classic acridine orange assay supported that HA-mediated sperm selection, by the PICSI dish, is an optimal tool for ICSI sperm selection.

Furthermore, in a more refined experiment, semen aliquots were smeared on glass slides, and the sperm were fixed for the various markers. Another aliquot of the same semen was incubated on hyaluronic acid-coated slides. After the 15-min binding process, the unbound sperm were gently rinsed off, and the HA-bound fraction was fixed. In the semen sperm fraction, there were sperm with cytoplasmic retention, DNA degradation (detected in individual spermatozoa with DNA-nick translation), aberrant shape, and persistent histones with aniline blue staining, whereas in the HA-bound sperm fraction, there was no presence of sperm with any of the cytoplasmic, nuclear, or shape defects [3].

Aneuploidy Frequencies in the HA-Bound Spermatozoa

Regarding the HA-mediated ICSI sperm selection, in addition to the DNA integrity, there is now focus on the increase in chromosomal aneuploidies with the embryologist-selected sperm, as the aneuploidy frequencies are 3–4 times higher in the ICSI offspring. Pointing out the relationship between meiotic and late spermiogenetic events, it was shown that sperm with cytoplasmic retention defects and sperm dysmaturity also have increased frequencies of chromosomal aneuploidies with a significant correlation at the level of $r > 0.75$, $p < 0.001$ [77]. Thus, these data, along with the below discussed experiments by Jakab et al. with FISH studies of >20,000 spermatozoa [58], suggest that the filtering effect of

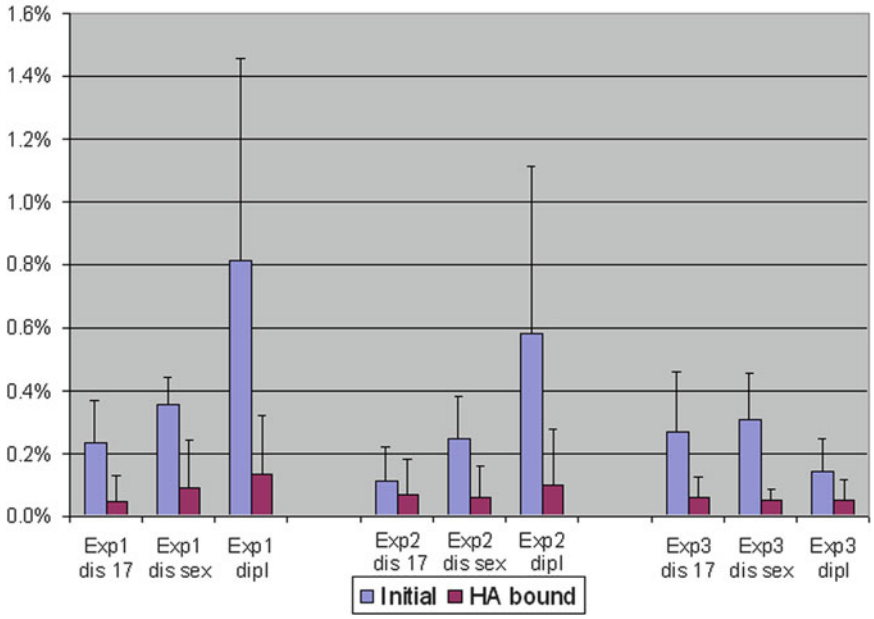

Fig. 27.8 Aneuploidy frequency results after the three experiments (experiments 1–3). *dis* disomy; *dis sex* sex chromosome disomy; *dipl* diploidy (from Jakab et al. [58], with permission)

the zona pellucida has been reconstructed and tested by hyaluronic acid binding. No matter how high the aneuploidy frequency was in the initial semen sperm fraction, in the sperm bound, and removed from HA, there was a 4–6× decline in disomy and diploidy frequencies to the 0.1–0.2% normal range, which is customary in babies conceived with natural conception or with conventional IVF (Fig. 27.8). Thus, the clinical use of HA-mediated sperm selection could ultimately solve the pertinent problem of ICSI with increased frequency of aneuploidies in the offspring when the ICSI is performed with embryologist-selected sperm from samples with high proportion of dysmature spermatozoa which can be detected by the score of the sperm-HA binding assay.

ICSI Sperm Selection by HA-Binding: FISH Analysis of Sperm in Semen and in the Respective HA-Selected Sperm Fractions

The increased rate of chromosomal aberrations and other potential consequences of using immature sperm for ICSI are of major concern [78–81]. Based on the presence of the HA receptors in mature, but not in dysmature sperm, coupled with a respective device with an HA-coated surface, we expected that the method will facilitate the selection of single mature sperm with high DNA integrity and low frequencies of chromosomal aneuploidies for ICSI. As demonstrated (Fig. 27.6), the HA-selected mature sperm are devoid of cytoplasmic retention, persistent histones, and DNA fragmentation.

Regarding ICSI sperm selection, we have tested the efficiency of elimination of aneuploid and diploid sperm from the HA-bound population [58] in three experiments. The sperm selection studies utilized Falcon Petri dishes with HA spots for ICSI sperm selection, so-called PICSI dishes (Biocoat Inc., Fort Washington, PA).

A drop of washed sperm was placed close to the edge of the HA spot, and the sperm were allowed to spontaneously migrate. The mature sperm that had completed plasma membrane remodeling bound to the HA, while arrested maturity sperm with lower HA receptor concentrations moved freely over the HA (Fig. 27.8, left). The HA-bound sperm also exhibited vigorous beating with increased tail cross-beat frequency [39, 40, 52]. After 15 min, the HA-bound sperm were collected with the ICSI micropipette (Fig. 27.7, right), fixed with methanol-acetic acid, and subjected to FISH, using centromeric probes for the X, Y, and 17 chromosomes [58].

In the control semen fraction of 34 men we analyzed a mean of 4,770 sperm, or 162,210 sperm in all. In the HA-bound and micropipette collected sperm fractions, due to the burdens of the task, there were fewer sperm studied.

In the first experiment, 12 moderately oligozoospermic men (sperm concentration: $20.6 \pm 1.7 \times 10^6$/mL, motility: $52.1 \pm 2.5\%$, all data mean ± SEM) were examined based on 7,530 sperm (range: 224–1,142 per man). In the HA-selected sperm vs. initial semen with the exception of Y disomy, the frequencies of all other aneuploidies and diploidies declined: Fourfold for 17 disomy, 5.7-fold for sex chromosome disomies, and 6.2-fold for diploidies. Indeed, no matter how high the frequencies were in semen, the HA-selected sperm were within the range of normozoospermic men (Fig. 27.8, experiment 1).

In the second experiment (12 normozoospermic patients, sperm conc.: $121.3 \pm 21.4 \times 10^6$/mL, motility: $59.5 \pm 4.9\%$), we addressed the question of whether HA selection would

cause a decline in chromosomal aberrations even in sperm fractions of normozoospermic samples that were further enhanced in mature sperm via gradient centrifugation about 9,720 sperm (range: 373–1,955 sperm per man). As expected, in the normozoospermic samples, the frequencies of disomies and diploidies were lower compared with those of oligozoospermic men (Fig. 27.8, experiment 2). However, HA binding resulted in a substantial selection effect, as there was a 4.3-fold decline in sex chromosome disomies and a 5.8-fold reduction in diploidies. As in experiment 1, the frequencies of individual aneuploidies or diploidies were in the very low 0.04–0.1% range.

In the third experiment, ten oligozoospermic men were studied (sperm concentration: $12.6 \pm 1.2 \times 10^6$/mL, motility: 49.3 ± 4.0%) in a setting similar to IVF-ICSI laboratories where the embryologist collect the HA-bound sperm individually. In the *third experiment* of individually selected sperm, 24,420 sperm were evaluated. In this approach, which required marathon collection sessions, we studied a mean of 2,442 HA-selected sperm in each of the 10 men (range: 1,086–3,973). As Fig. 27.8, experiment 3 indicates, neither the frequencies of disomies nor the significant reduction in disomy frequencies in the HA-selected sperm differed from those produced in experiments 1 and 2.

Overall Results

In the HA-bound sperm vs. unselected sperm, the chromosomal disomy frequencies, with the three probes studied, were reduced to 0.16 from 0.52%, diploidy to 0.09 from 0.51%, and sex chromosome disomy to 0.05 from 0.27% (a 5.4-fold reduction vs. a 4–5-fold respective increase in ICSI offspring). No matter how high the aneuploidy frequencies in the semen sperm fractions were, the respective frequencies were within the narrow low 0.04–0.10% range per probed chromosome in HA-bound sperm, comparable to the range of normozoospermic fertile men. The fivefold decline in X, Y, and XY disomies is consistent with the increase in chromosomal aberrations in ICSI children conceived with visually selected sperm [80, 82].

Although the primary ICSI candidates are men with severe oligozoospermia (notwithstanding the fact that success-oriented IVF laboratories use ICSI almost exclusively, thus almost any couple may be exposed to the ICSI risks), it was necessary to use oligozoospermic and borderline oligozoospermic samples in order to collect sufficient numbers of spermatozoa to statistically validate the experimental results. However, because ICSI sperm selection reflects the maturity status and binding of single sperm, we believe that *HA-bound mature sperm* of a severely oligozoospermic man or of men with higher sperm density should not be different. If a sperm binds to HA, it will bind whether there are ten or thousands of mature or dysmaturity sperm in the drop. Thus, due to the polymorphic nature of sperm, similar to the zona pellucida binding, HA selections of mature sperm are likely to be independent from seminal sperm concentrations. This hypothesis is well supported by the sperm-HA binding studies, and by the three aneuploidy experiments, in which the aneuploidy frequencies in the HA-selected sperm fractions were similar and within a narrow, low, and normal range (Fig. 27.8).

Methods of HA-Mediated ICSI Sperm Selection Using PICSI Dishes

The methods of PICSI-mediated sperm selection maybe summarized as follows:

1. Sperm preparation is normally performed by using density gradient protocol (45 and 90% Isolate). A PICSI dish (modified Petri dish) has orienting lines, each ending with round HA-coated spot, in order to facilitate the approach to sperm selection. A 5–10 μL of washing media (modified human tubal fluid with 10% human serum albumin) is added to each ring at 37°C, under sterile conditions, and covered up with oil.
2. Thereafter, 1–5 μL of sperm suspension prepared in fertilization media (the suspension is ready for ICSI) should be placed into the media drop around the PICSI HA spot. (The volume of the sperm suspension will depend on the motile sperm concentration in the suspension; the lower the motile sperm concentration, the more sperm suspension media is needed).
3. The incubation with the PICSI dishes is carried out for 5–15 min at 34–36°C (Fig. 27.9). (The PICSI dish has some temperature-filtering effect; thus, alteration of the stage temperature should not be mandatory. During the observation time, one can clearly distinguish between spermatozoa which is normally developed, underwent the plasma membrane remodeling and bind to the HA spot, as well as they exhibit a higher tail cross-beat frequency or the dysmature spermatozoa that do not perceive the HA, and swims along).
4. Following the observation step, the PICSI dish is moved to the micromanipulation microscope. At this time, ICSI dishes should be ready to put the collected sperm from the PICSI dishes. Sperm should be collected using ICSI (microinjection) needle. In 5–15-min incubation time, there will be enough sperms attached to the HA spot. Using ICSI needle, 10–100 of attached sperm should be collected. The embryologist should prefer to pick up and collect sperm with the fastest tail cross beat frequency. This is important because of the sperm with faster tail cross-beat frequency have a more enhanced HA receptor density, indicating that the sperm membrane remodeling was more effective.
5. The sperm are collected from the PICSI HA spot with an ICSI needle and are ejected into a sperm ICSI washing media. This will be followed by the routine procedure of ICSI injection with the collected sperm.

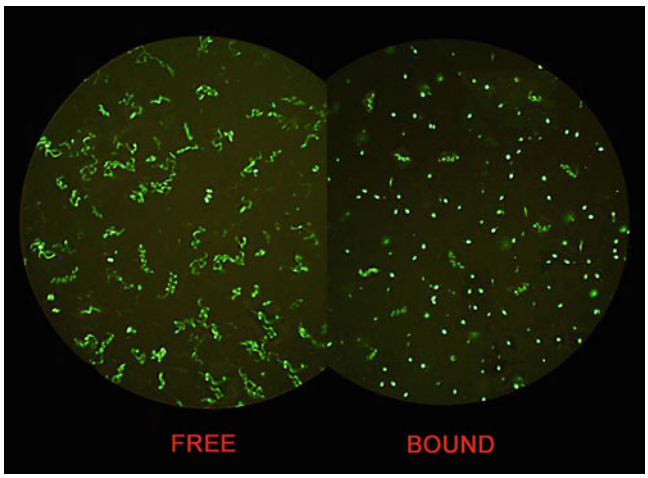

Fig. 27.9 Selection of mature spermatozoa for intracytoplasmic sperm injection (ICSI). (**a**) Time-lapse photography. Spermatozoa applied to the periphery of an HA spot. Normally developed spermatozoa bind to HA (*solid spots*) and maintain their tail-beating activity, whereas dysmature spermatozoa, lacking HA receptors, freely proceed over the HA coating. (**b**) After removal of the unbound spermatozoa by gentle rinsing, the normally developed, HA-bound, spermatozoa are selected with the ICSI pipette (from Huszar et al. [3], with permission)

IVF-ICSI Data with HA-Mediated Sperm Selection

There are now several laboratories that have initiated use of HA-mediated sperm selection. It is important that none of the groups that practice the HA sperm selection reported any adverse effect on fertilization or embryo development. In one 2005 ESHRE presentation, data on 18 pregnancies were reported. Comparing ICSI results with *visually selected ICSI sperm* (*N* = *84*) vs. *HA-selected ICSI sperm* (*N* = *18*): fertilization rates: 69.7% vs. 67.0%; good grade embryos: 56.6% vs. 51.7%; pregnancy rates: 45.3% vs. 33%; miscarriage rates: 7% vs. 0%; take-home baby rates: 46.5% vs. 39.0% [83].

Another related 2006 study was performed by the Brussel group on 20 unselected couples treated with ICSI in which at least eight MII oocytes were recovered. The sibling oocytes were injected either with HA-selected or with visually selected spermatozoa (*N* = 146 and 145). The fertilization rates (72.9 and 66.9%), oocyte degeneration rates (9.6 and 13.8%), rate of embryo cleavage (cell numbers), embryo quality, embryo transfer, and embryo cryopreservation rates were all similar. The authors suggest that in addition to the sperm selection utility in ICSI, the use of HA-selected sperm may be useful in patients undergoing preimplantation genetic screening in which the chromosomal status of the embryos can be related to the sperm selection technique [84].

In another relevant publication [85] improvements with HA-selected sperm were reported in porcine IVF. Porcine embryos were produced by IVF, ICSI, and ICSI performed with HA-selected sperm. The HA-mediated sperm selection was superior to visual sperm selection in producing chromosomally normal embryos and increasing ICSI efficiency by reducing the early embryonic mortality and thus enhancing ICSI success rates.

A number of recent studies have indicated that HA-bound sperm used in the ICSI procedure (the so-called physiological ICSI) may lead to increased implantation rates. In one such study, Parmegiani et al. [86] showed that in 293 couples treated with HA-ICSI vs. 86 couples treated with conventional ICSI (historical control group), all outcome measures of fertilization, embryo quality, implantation, and pregnancy were the same or improved in the HA-bound sperm group [86]. The implantation rate was increased from 10.3% in conventional ICSI to 17.1% in the HA group. The authors concluded that if multicenter randomized studies confirm the beneficial effects on ICSI outcome, HA could be considered as a routine choice for "physiologic" sperm selection prior to ICSI. A smaller clinical trial assessing the same technology by Worrilow et al. has also shown that clinical pregnancy rates are improved when using HA-selected sperm compared to conventional ICSI [87]. Furthermore, the sperm-HA binding score (the proportion of sperm that underwent plasma membrane remodeling in spermatogenesis and binds to hyaluronic acid) is an important diagnostic indicator. Men with <55% binding score would particularly benefit, as their ICSI success rates were improved (20–30% higher pregnancy rates) by using the HA-mediated sperm selection [87]. Thus, it is important that in IVF programs, the Andrology laboratory performs the sperm-HA binding test for the husbands of IVF-ICSI couples, based on the HA-binding score which provides the information on the proportion of sperm with attributes of dysmaturity, such as DNA fragmentation, chromosomal aneuploidies, persistent histones, cytoplasmic retention, and lack of HA-binding ability, consequential to dysmaturity. The IVF team triages the couples according to their HA-binding score in order to perform conventional IVF or ICSI. Another aspect of the markers of sperm development is the enhancement of sperm with normal morphology in the HA-bound sperm fraction. Studies in the Huszar laboratory indicated that there was a 2–3-fold enrichment of Tygerberg normal morphology sperm compared to the respective semen sperm fraction, which, interestingly, agreed with the finding of the Tygerberg group with respect to the enrichment of normal morphology sperm in the zona pellucida bound vs. semen sperm fraction [56, 57].

In a more recent 2006 study of 26 patients (273 oocytes retrieved and 177 embryos created by ICSI), the fertilization rates with visually selected and HA-selected sperm were similar (66.6 and 61.1%). Among the 22 couples with embryo replacement, 8 couples received embryos fertilized with visually selected sperm, 7 couples received embryos with HA-selected sperm, and another 7 couples received embryos of both origins. The respective pregnancy rates were 25, 57.1, and 57.1% with significantly higher rates in the two

groups that received HA-selected embryos. Regarding miscarriage rates, they were higher in the group that received visually selected embryos ($p=0.013$) [88]. All of these data were confirmed by a recent larger multicenter study, comparing ICSI outcomes with eye-selected and HA-selected spermatozoa which was presented at recent meetings by Worrilow et al. [87–88]

Other ICSI Sperm Selection Methods

Sperm Selection by Sperm Charge Properties

The Aitken's group recently reported a novel *electrophoretic sperm isolation* device utilizing a separation strategy based on sperm size and electronegative charge. The suspensions generated by the electrophoretic separation technique contained motile, viable, and morphologically normal spermatozoa while exhibited low levels of DNA damage. Reportedly, the electrophoretic sperm isolation procedure is both time and cost-effective [89], and the first pregnancy using this method for a couple suffering from extensive sperm DNA damage was reported [90]. Further, in a clinical trial of IVF and ICSI fertilization with gradient prepared sperm and electrophretically prepared sperm, the resulys were compbarable with repect to fertilizarion rates, cleavage and embryu quality [113].

Intracytoplasmic Morphologically Selected Sperm Injection

In 2001, Bartoov et al. [91] reported the selection of spermatozoa with normal nuclei to improve the pregnancy rate with intracytoplasmic sperm injection. They further verified this technique by performing ICSI using morphologically normal sperm, strictly defined by high power light microscopy (×>6,000). Sixty-two couples, with at least two previous consequent pregnancy failures after ICSI, underwent a single ICSI trial preceded by morphological selection of spermatozoa with normal nuclei. Fifty of these couples were matched with couples who underwent a routine ICSI procedure at the same IVF center and exhibited the same number of previous ICSI failures. The matching study revealed that the pregnancy rate after modified ICSI was significantly higher than that of the routine ICSI procedure (66.0% vs. 30.0%).

More recently, Antinori et al. conducted a prospective randomized study to assess the advantages of intracytoplasmic morphologically selected sperm injection (IMSI) over the conventional ICSI procedure [92]. A total of 446 couples with 3 years of primary infertility, the woman aged 35 years or younger, without workup for female factor, and husbands of severe oligoasthenoteratozoospermia were randomized to eye-selected ICSI ($N=219$; group 1) and IMSI ($N=227$; group 2) treatment groups. The data showed that IMSI resulted in a higher clinical pregnancy rate (39.2% vs. 26.5%; $p=0.004$) than ICSI.

The use of IMSI appears promising [93]. Some drawbacks are however present, in particular, the belief that it is a complicated technique that cannot be routinely performed [94–96]. As the high-magnification approach is increasingly used, more studies will be focused upon the selected sperm, their respective levels of cytoplasmic retention, persistent histones, DNA chain integrity, aneuploidy rates, tyrosine phosphorylation, or apoptotic markers should be performed in order to verify the safety with respect to the potential public health concerns.

Potential Benefits of the HA-Mediated Sperm Selection Method for the ICSI Offspring

The HA-mediated ICSI sperm selection, by introducing only mature spermatozoa, will maintain the genetic impact and paternal contribution of sperm to the zygote at the traditional evolutionary level. Thus, the HA method may alleviate the potential problems related to chromosomal aneuploidies and DNA chain fragmentation that presently cause concern due to ICSI fertilization with visually selected sperm that may be of arrested maturity [9, 31, 35, 49, 52–54, 78, 97]. The safety of HA-mediated sperm selection is supported by various lines of evidence. First, HA occurs normally in the female reproductive tract and in the cumulus oophorus. Thus, it is likely that HA is carried with the sperm into oocytes even during in vivo conception. In the case of ICSI, the removal of sperm from HA may cause a few HA molecules to attach to sperm or a fraction of μm^2 of sperm membrane from the area of the acrosomal cap (which is lost otherwise during the acrosomal reaction) to remain attached to the HA.

DNA damage is associated with a decline in pregnancy rates following natural conception, but it has also been linked to diminished success in assisted conception due to reduced rates of fertilization, disrupted development of the zygote, and early pregnancy loss [4, 50, 54, 98, 99]. This is an increasingly important factor because 3–6% of the populations in developed countries now utilize assisted conception for reproduction. Another, related, concern is raised by the relationship between paternal and maternal (in pregnancy) smoking and other associated oxidative damage to DNA in response to xenobiotics, pesticides, and environmental chemicals which may promote testicular cancer and childhood cancer [50, 100–102].

Arrested maturity, or dysmature sperm with DNA damage and arrested membrane remodeling, that is unable to fertilize in natural conception is likely be eliminated by HA-mediated ICSI sperm selection with potential improvements in various areas: (a) Following ICSI fertilization with visually selected sperm, there were increased rates of de novo numerical chromosomal aberrations and also cytogenetically detectable structural chromosomal aberrations. These are most likely due to increased rates of chromosomal aneuploidies, primarily sex chromosome disomies, in sperm of ICSI fathers

[79–82, 103]. (b) With visually selected sperm and ICSI, there is a reportedly higher incidence of spontaneous abortions in the 18% range, compared to the 10% rate following normal conception [81]. (c) In a recent multicenter study from five European countries which focused upon 5-year-old singleton children, a potentially increased risk of birth defects was reported; the odds for malformations were 2.77 for ICSI vs. naturally conceived children ($N=540$ and 538, respectively) [79]. (d) Men treated with ICSI also show a higher rate of chromosomal rearrangements, such as reciprocal and Robertsonian translocations. These rearrangements may be associated with oligozoospermia and infertility, as well as, via interchromosomal effects, disomies and diploidies. Thus, the HA-mediated sperm selection for ICSI may reduce the risk for the chromosomal aberrations for the offspring, if a man has a common origin of chromosomal rearrangements, numerical aberrations, and arrested sperm maturity [104–106]. (e) Regarding structural chromosomal abnormalities in ICSI-derived pregnancies after visual sperm selection, the incidence of abnormal karyotypes was examined via chorionic villus sampling and amniocentesis. In 1,586 subjects, there were 47 children (3%) with abnormal fetal karyotypes, and 25 of these (1.6%) were de novo. Regarding the role of sperm maturity, the frequency of structural chromosomal abnormalities was approximately tenfold higher (24/1,120 or 2.1% vs. 1/1,419 or 0.24%, $p=0.006$) following ICSI fertilization by oligozoospermic and severely oligozoospermic men [107]. (f) In line with the idea of the Brussels group who see a potential for fertilization with HA-selected sperm for couples who plan to undergo preimplantation genetic diagnosis or preimplantation genetic haplotyping, fertilization with HA-selected mature sperm may delineate into the oocyte aneuploidies and chromosomal aberrations found in embryos. Data supporting this approach arising from recent studies [108, 109]. (g) Using HA-selected mature sperm may increase pregnancy success following ICSI by reduction of sperm-derived aneuploidies and defects of oocyte activation that lead to early embryo failure [110, 111].

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Electrophoretic Sperm Separation

28

Steven Fleming and John Aitken

Abstract

By virtue of its ability to rapidly isolate spermatozoa with good morphology and low levels of DNA fragmentation, electrophoretic sperm separation promises to be an extremely versatile, time-saving and efficient method for preparing spermatozoa for a wide variety of applications in assisted reproduction.

Keywords

Electrophoresis in sperm separation • Sperm separation • Electrophoretic technology for sperm sorting • Sperm sorting technology • Sperm separation equipment

Principles of Electrophoresis

Electrophoresis is a term used to define the motion of a particle 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.

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 [5]. 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].

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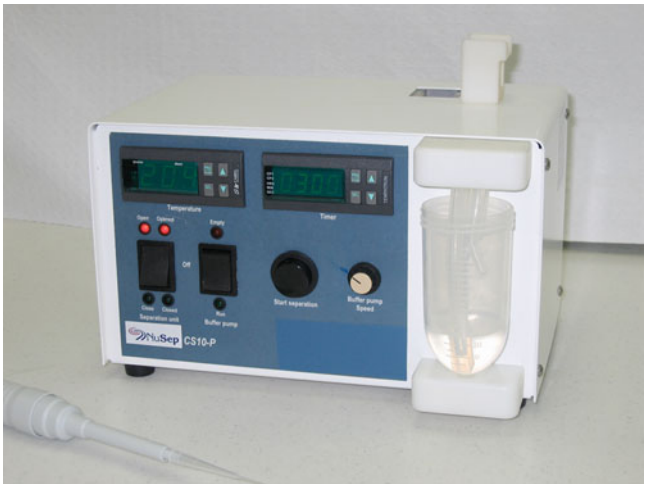

Fig. 28.1 The prototype CS10 instrument

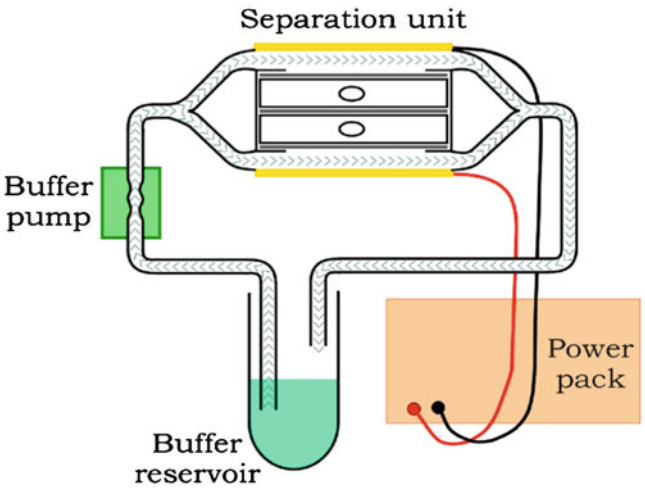

Fig. 28.2 Diagrammatic representation of the CS10 design

Development of Electrophoretic Technology for Sperm Sorting

The life separations company, NuSep, has been concerned with the development of bioseparations products for the past 30 years. In collaboration with Prof. John Aitken at the University of Newcastle, NuSep further developed their laboratory protein separations instrument, the ProteomeSep MF110, to create a prototype instrument designed for sperm separation, called the cell sorter 10 (CS10; Fig. 28.1). The CS10 was based upon preparative isolation by membrane electrophoresis (PrIME) technology, 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 of NuSep, called SpermGen, is developing the CS10 into a regulatory compliant production unit, known as the SpermSep CS10.

The CS10 applies an electric potential via platinum-coated titanium mesh electrodes to move 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 towards 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].

Equipment Setup and Separation Parameters

Separation Cartridges and Sample Handling

The separation cartridge of the prototype CS10 is a self-assembled device that has either a symmetric or an asymmetric format. In the asymmetric design, the inoculation or loading chamber has a 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, providing that the prostaglandins present within seminal plasma have been removed or reduced to clinically insignificant levels. However, for the purposes of in vitro fertilisation (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 20×15 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).

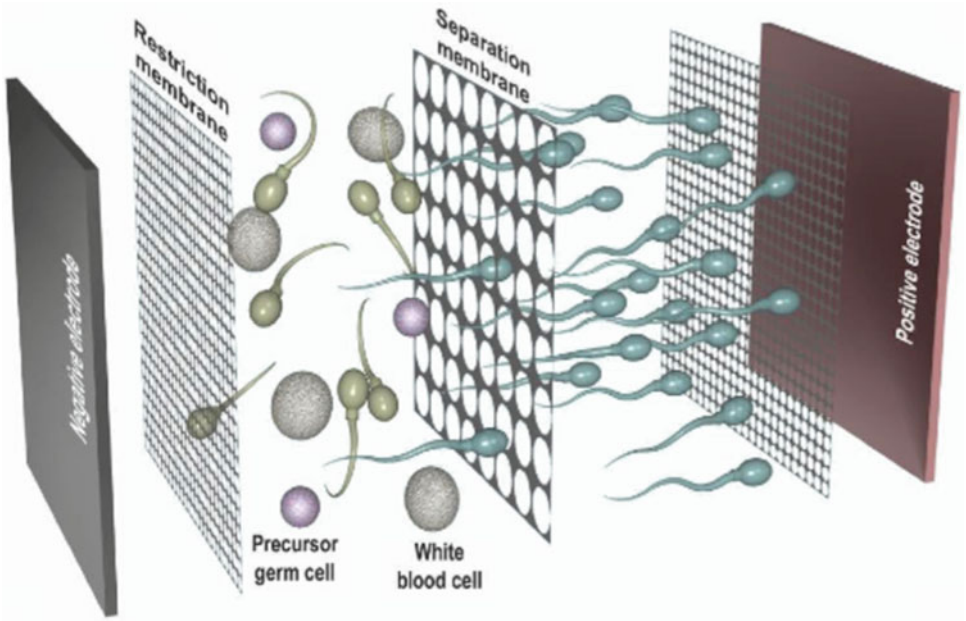

Fig. 28.3 Schematic diagram showing sperm electrophoretic mobility

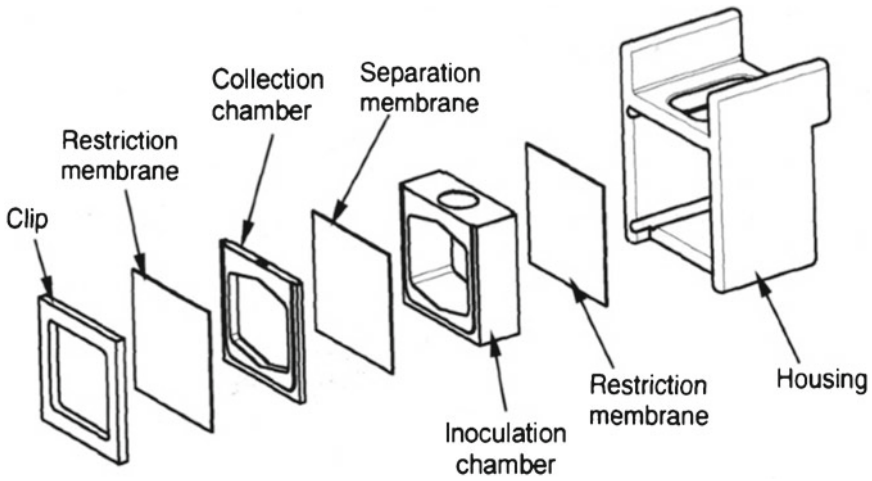

Fig. 28.4 Exploded diagram of the asymmetric separation cartridge

The separation cartridge is inserted into the cartridge housing on top of the SpermSep 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 mechanism is activated, the cartridge components are made watertight by the machine sealing pressure applied by the SpermSep CS10. 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, 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.

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-sterilised prior to use with a 0.22-μm filter (Millipore Corp., Bedford, MA, 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

on the front of the SpermSep CS10 (Fig. 28.1). In order to prevent overheating during operation of the instrument, the buffer is maintained at 25°C and is circulated round 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.

Current and Voltage Settings

The input power specifications of the SpermSep CS10 are 115–240 V at 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.

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 SpermSep 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.

Method Validation

Initial validation of the SpermSep 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].

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 30 s 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].

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.

Sperm Morphology and DNA Integrity

The percentage of normal spermatozoa observed following staining by a modification of the Papanicolaou method [12] and assessed using the sperm deformity index (SDI) [13] 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 fertilisation potential [13].

DNA damage, assessed using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (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.

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 fertilise the oocytes in this instance, it was still unknown whether electrophoresis might compromise aspects of sperm function

necessary for normal fertilisation. This uncertainty was resolved following a prospective, split-sample, split-cohort controlled clinical trial, involving patients having both ICSI and IVF, with sperm prepared either by 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 fertilisation (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 [14–16].

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 ($28.4 \pm 7.1\%$).

Closing Remarks

Combined, 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 SpermSep CS10. 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].

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Magnetic-Activated Cell Sorting of Human Spermatozoa

29

Enver Kerem Dirican

Abstract

Magnetic cell sorting allows large numbers of viable cells to be isolated with high purity and yield. It is accepted that the process does not cause physical damage to the cell. Today, magnetic nanoparticles are widely used in separation of macromolecules or cells from various cell suspensions. Studies have shown that nonapoptotic sperm fractions selected by MACS technique have morphologically superior quality, higher percentage of motility, viability, improved apoptosis indices and routine sperm parameters. Reduction of apoptotic spermatozoa within the ejaculate by means of the MACS system results in a distinct reduction of spermatozoa with DNA fragmentation. The initial clinical trials of MACS of human spermatozoa in assisted reproduction showed a statistically significant improvement in the embryo cleavage rates.

A number of studies, including our own, have shown that using spermatozoa prepared with MACS technique significantly improves the quality of spermatozoa in the preparation. The efficiency of this new technique is still not fully evaluated, and the interpretation of ICSI results still remains incomplete. More research is needed to improve our current knowledge in relation to human sperm apoptosis and MACS technique.

Keywords

Magnetic-activated cell sorting • Sperm apoptosis • Assisted reproduction • Density gradient centrifugation • Swim-up • Sperm DNA fragmentation

Magnetic Nanoparticles in Cell Separation

Magnetic Nanoparticles Are Highly Biocompatible

The history of cell separation is long, dating back to the 1950s and 1960s [1, 2]. Since then, the success in the synthesis of magnetic particles has empowered a plethora of exciting biotechnological applications [2].

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Magnetic cell sorting allows large numbers of viable cells to be isolated with high purity and yield [3]. The superparamagnetic microbeads used in magnetic cell separation are amorphous or semicrystalline structures [2] and are small enough (20–100 nm) to be colloidal, i.e., they remain dispersed due to the random bombardment of Brownian motion. The magnetically susceptible core is small enough that the particles do not retain any residual magnetism when removed from the field. These properties facilitate both the preparation of antigen-specific reagents and separation of specific cell types [3].

In the context of the emerging concern about the potential toxicity of nanoparticles, it is noteworthy that magnetic iron oxide particles are highly biocompatible, as the iron cell homeostasis is well controlled by uptake, excretion, and storage, and the iron excess is efficiently cleared from the body [2]. Although there are few reports on the negative effects of

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magnetic fields in male reproduction and semen quality [4], several studies have been carried out, and none of them identified any decreased fertility for either males or females [5]. And it is accepted that the process does not cause physical damage to the cell and the magnetic particles do not affect the rate of growth of cell cultures [6]. Therefore, even introduction of these particles inside a living cell is considered as a viable process [3, 6].

Magnetic Nanoparticles Are Widely Used in Research and Clinical Protocols of Various Biomedical Disciplines

Magnetic separation has been successfully applied to many aspects of biomedical and biological research. It has proven to be a highly sensitive technique for the selection of rare tumor cells from blood and is especially well suited to the separation of low numbers of target cells [7]. This has, for example, led to the enhanced detection of malarial parasites in blood samples either by utilizing the magnetic properties of the parasite [8] or through labeling the red blood cells with an immunospecific magnetic fluid [9]. It has been used as a preprocessing technology for polymerase chain reactions, through which the DNA of a sample is amplified and identified [10]. Cell counting techniques have also been developed using magnetic technologies [11, 12].

Today, magnetic nanoparticles are widely used in separation of macromolecules or cells from various cell suspensions or homogenates [2] and also in protein purification [13] and nucleic acid applications [14].

Magnetic cell sorting is widely used in many clinical areas in cellular therapies [15] including human autoimmunity diseases like rheumatoid arthritis [16], diabetes [17], multiple sclerosis [18], and systemic lupus erythematosus [19], in nucleic acid transfer as a transfection method [20] and to optimize conditions for viral-mediated gene delivery (therapy) by magnetofection [21].

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 [22] and for elimination of apoptotic spermatozoa from human semen samples [23–25].

Apoptosis and Reactive Oxygen Species in the Human Spermatozoa

Defining the "Good" Sperm

In the era of human assisted reproduction where the main choice of treatment for the severe male infertility is intracytoplasmic sperm injection (ICSI), quality of gametes is one of the factors that help to determine the success.

With the innovation of strict Tygerberg criteria, a consensus regarding the importance of sperm morphology assessment in human assisted reproduction seems to be reached. After the breakthrough in human assisted reproduction by ICSI, the raised question about the future need of extended sperm analysis as well as sperm function tests evoked [26, 27].

Infertile men with poor sperm motility and morphology were found to have increased sperm DNA fragmentation compared with individuals with normal semen parameters [28]. 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 [29], defective apoptosis before ejaculation [30], or excessive production of reactive oxygen species (ROS) in the ejaculate [31]. Exposures to environmental or industrial toxins [32], genetics [33], or lifestyle [34] 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 organelle is the sperm nucleus. Studies reveal that severe teratozoospermia results in high preimplantation embryo aneuploidy [35, 36] and the interchromosomal effect is related to impaired semen parameters [37]. Studies have also shown that immature sperms have increased rates of lipid peroxidation and bear poor morphometric and morphological attributes, zona pellucida-binding properties, and fertility [38]. 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 caspases-3 activation and mitochondrial membrane potential integrity [39].

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 [40]. Table 29.1 shows common characteristics of a *good* sperm.

Why Do Reproductive Technologists Need to Select Sperm Prior to Assisted Reproduction?

The spermatozoa of all placental mammals, including humans, are in a protective state at ejaculation and are incapable of fertilization. Spermatozoa must undergo a subsequent period of final maturation termed capacitation, and

Table 29.1 Common characteristics of a *good* sperm for ICSI

Feature	Desired	Undesired
Morphology	Classified as "normal" in Tygerberg criteria	Vacuoles, elongation, acrosomeless forms, megalo, or duplicated
Motility	Progressive	Immotile, agglutinated
Maturity	Fully matured, not aged	Cytoplasmic droplets, short tails
Functionality	Hypo-osmotic tail swelling (HOS) present	HOS absent
Biochemistry	Low ROS (reactive oxygen species)—high AC (antioxidant capacity)	High ROS—low AC
Nucleus	DNA not fragmented, euploidy	DNA fragmented, aneuploidy
Cellular viability	Nonapoptotic, viable	Apoptotic, necrotic

spermatozoa in the ejaculate are prevented from undergoing capacitation by some factors that are present in the seminal plasma [41]. This final maturation of the mammalian spermatozoa can be evaluated in three steps: capacitation, hyperactivated motility, and acrosome reaction.

As a common sense, sperm preparation prior to assisted reproduction has three basic advantages:

1. Sperm preparation and wash procedures remove the seminal plasma and help the sperm cells to prepare for capacitation.
2. Sperm preparation methods help to eliminate immotile, dead, and morphologically abnormal spermatozoa and nonreproductive cells, which are the main causes of ROS in the ejaculate.
3. Sperm washing and selection techniques can be used to purify the sperm cells from pathogenic microorganisms.

Since the oocyte selects the best sperm to win the race in natural conception and conventional insemination protocols like intrauterine insemination (IUI) and in vitro fertilization (IVF), the contribution of the healthy paternal genome to embryo development, implantation, and conception is the main problem to be solved in sperm preparation for ICSI.

Current Sperm Preparation Techniques and Limitations in Assisted Reproduction

First, sperm preparation technique was washing the spermatozoa with culture medium followed by centrifugation and resuspension of the pellet. Today, there are a number of different sperm preparation methods available, and they are concentrated on selecting sperms with normal morphology, normal and intact acrosomes, and progressive motility.

Density Gradient Centrifugation

According to the principles of density gradient centrifugation (DGC), when a suspension of particles is centrifuged, the sedimentation rate of the particles is proportional to the force applied. At a fixed centrifugal force and liquid viscosity, the sedimentation rate is proportional to the size of the particle and the difference between its density and the density of the surrounding medium. DGC method uses these principles to select motile sperm with good morphology and a healthy chromatin structure [42]. Although some studies point out that the gradient solutions may damage sperm [43], generally the technique is considered to recover sperm with good morphology and high motility [44].

Swim-Up

Swim-up is the choice of semen processing for IUI and IVF, in cases where no male factor is present to mild male factor infertility. The swim-up from a washed pellet method is the most common version of the technique, and it is characterized by extremely high proportions of motile spermatozoa, a preferential selection for morphologically normal spermatozoa, and an absence of the other cell types and debris commonly seen in human ejaculates [45].

As the indications for IVF treatment were expanded beyond simple tubal factor cases to couples with idiopathic infertility and, ultimately, to male factor cases, the problem of fertilization failures [46] and insufficient motile sperm yields appeared by swim-up techniques.

Other Techniques

A variety of techniques were used in human assisted reproduction for the improvement of selecting the best sperm. Most of these techniques are not in routine clinical use anymore, like Percoll gradients, Nycodenz gradients, affinity chromatography, glass beads, sephadex columns, transmembrane migration, etc.

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 ICSI [40]. These include the use of high-magnification microscopes to identify minor morphological defects of the living spermatozoa [46], selection of mature spermatozoa referring to the binding ability of human spermatozoa to hyaluronic acid [47], and eliminating apoptotic spermatozoa prior to ICSI by magnetic-activated cell sorting (MACS) [48].

Principles of Magnetic Selection in Human Spermatozoa

Programmed cell death in animals usually occurs by apoptosis. Cells dying by apoptosis undergo characteristic morphological changes. They shrink and condense, the cytoskeleton

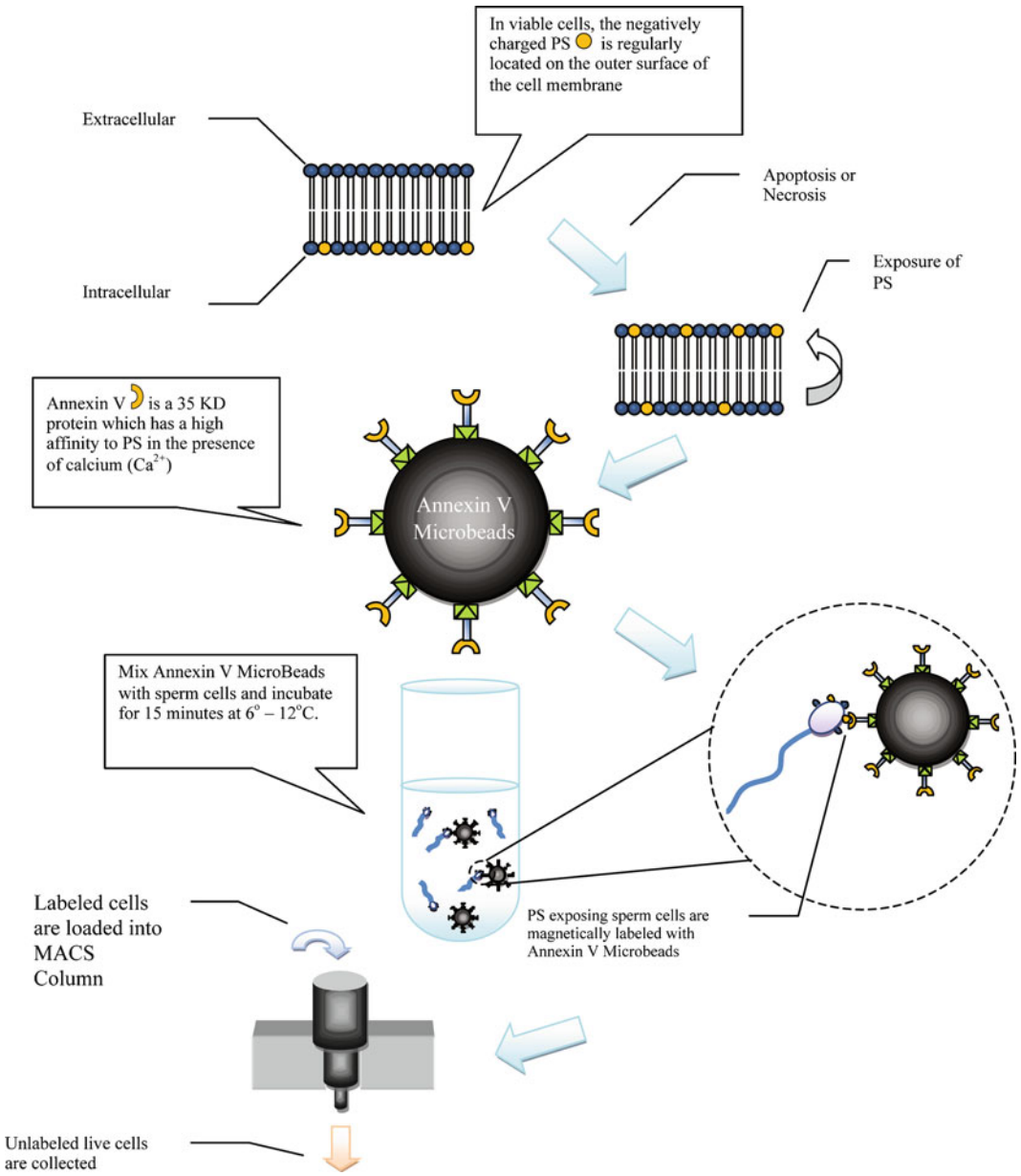

Fig. 29.1 The Annexin V-coated microbeads are used for the isolation of cells with exposed PS, including sickle cells and apoptotic cells, and removal of dead and apoptotic spermatozoa from seminal samples. Briefly, the PS-exposing spermatozoa are magnetically labeled by the protein Annexin V, then the magnetically labeled apoptotic and dead spermatozoa are retained on a MACS column where the unlabeled nonapoptotic 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)

collapses, the nuclear envelope disassembles, and the nuclear chromatin condenses and breaks up into fragments. 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 [49].

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, 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 [49].

The Annexin V-coated microbeads are used for the isolation of cells with exposed PS, including sickle cells and apoptotic cells, 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 MACS column where the unlabeled nonapoptotic spermatozoa are in the flow-through (Fig. 29.1). At the end, magnetically labeled, PS-exposing spermatozoa

can be eluted from the column for various cellular tests (Miltenyi Biotec GmbH, Germany).

Preliminary Studies on Magnetic-Activated Cell Sorting of Human Spermatozoa

Several studies using MACS technique with human spermatozoa have been published over the past 10 years. Interests in these studies were mainly the molecular efficiency of the technique and improving the postpreparation sperm quality.

Efficiency of Magnetic Selection in Conventional Seminal Parameters Was Shown in Some Studies

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 DGC is an effective sperm preparation technique that does not lead to significant cell loss and separating a distinctive population of nonapoptotic spermatozoa with intact membranes might optimize the outcome of assisted reproduction [50]. Later on, the separation effect of MACS on capacitation and acrosome reaction was investigated in the nonapoptotic sperm fractions. Nonapoptotic human spermatozoa with intact plasma membranes were found to be characterized by superior ability to capacitate and, consequently, by maximum potential to perform acrosome reaction after stimulation [51].

Studies have also shown that nonapoptotic sperm fractions selected by MACS technique have morphologically superior quality [52], higher percentage of motility, viability, and apoptosis indices [53] and routine sperm parameters [54].

Basic Studies Show Improvements in the Markers of Apoptosis and DNA Integrity

At the end of the 1990s, separation of leucocytes from human seminal plasma was successfully performed by magnetic selection [55]. 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 lysophosphatidylcholine content that is likely generated by phospholipases [56] and also are characterized by an increase in activated caspases, which were found in infertile patients [57].

Reduction of apoptotic spermatozoa within the ejaculate by means of the MACS system results in a distinct reduction of spermatozoa with DNA fragmentation [58], enrichment of spermatozoa free of apoptosis [59], and improvement of sperm viability, motility, and mitochondrial membrane integrity [60].

Cryobiology of Human Spermatozoa with Magnetic Selection Seems to Be Beneficial

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 [61]. 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 that MACS separation before cryopreservation results in depletion of spermatozoa which are positive for activated caspases [62], depletes low-quality spermatozoa from cryopreserved semen samples [63], and selects a population of nonapoptotic spermatozoa which optimizes cryopreservation and thawing outcome [64].

MACS from Research Bench to Clinical Application

The integrity of the paternal genome is of a paramount importance in the initiation and maintenance of a viable pregnancy in both natural and assisted reproduction. The need to eliminate nonviable and apoptotic spermatozoa before IVF is therefore one of the factors to improve the outcome of treatment for the infertile couple. Studies have investigated the sperm fertilizing potential using hamster oocyte penetration assay [65], and results suggested that nonapoptotic 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 [66]. Studies also showed a reduced level of apoptotic markers, improved acrosome reaction scores [67], and superior morphological quality [68] after MACS preparation which may contribute to increased implantation and pregnancy rates.

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 [69] as a preliminary study of a Turkish group.

A brief step-by-step protocol is as follows:

– Perform all steps under a laminar airflow 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 μL 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 an 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, 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 postseparation sperm values.
- Perform either a DCG or a swim-up preparation on the sperm suspension according to sperm concentration and motility.
- Use the prepared motile sperm cells for ICSI.

Human In Vitro Embryos Cleave Better After MACS

In the 2006 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 [69].

In 2007, a prospective study was designed in our program to assess the impact of MACS technique for selection of nonapoptotic spermatozoa on the outcome of ICSI [48]. We compared the cleavage, fertilization, implantation, and pregnancy rates associated with two sperm preparation methods, MACS and DGC, for the ICSI of superovulated women. Especially male factor cases were included in the study to define the effects of MACS preparation, and both MACS and DGC groups had comparable demographic characteristics. The magnetic enrichment of nonapoptotic spermatozoa significantly improved the percentage of sperm with normal morphology.

Although there were no significant differences between 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, although fertilization rates are not different from the control group, results in a significantly higher number of embryos on the embryo transfer day, by reducing the number of arrested embryos during in vitro culture [48].

Which embryos arrest in culture? Few studies address the issue in literature. Early embryo arrest is associated with the injection of round spermatids [70], pronuclear morphology, and chromosomal abnormalities [71, 72], which are possible deleterious factors that can be introduced into the human oocyte by spermatozoa [73, 74] and impair the implantation of that embryo. Therefore, it is acceptable to conclude that observed improvement in embryo cleavage rate in our study should be related to better elimination of apoptotic and abnormal spermatozoa by MACS technique.

Does Better Elimination of Apoptotic Spermatozoa Yield Higher Pregnancy Rates in Assisted Reproduction?

Our study demonstrates slightly higher clinical pregnancy and implantation rates after using MACS for preparation of human spermatozoa. Another study from Argentina reported three ongoing clinical pregnancies out of 10 cases by using MACS as a sperm preparation technique [75]. They also represented their first healthy baby born after MACS technique [76].

As apoptotic fractions represent highly abnormal spermatozoa, it should be expected that elimination of these cells would result in better pregnancy and implantation rates in human assisted reproduction.

Since the number of cases in both studies still remains low, further studies should be conducted to better characterize the process of eliminating abnormal spermatozoa and pregnancy rates.

The Future of MACS in Sperm Selection for Assisted Reproduction

A number of studies, including our own, have shown that using spermatozoa prepared with MACS technique significantly improves the quality of spermatozoa in the preparation. These studies indicate that MACS can enrich the sperm population by separating out apoptotic, necrotic, chromosomally abnormal, and DNA-fragmented spermatozoa [48,55-60].

Although our results showed improved sperm morphology and embryo cleavage rates after MACS, the efficiency of this new technique is still not fully evaluated, and the interpretation of ICSI results still remains incomplete. More research is needed to improve our current knowledge in relation to human sperm apoptosis and MACS technique.

Additionally, more standardized, large-scale clinical trials are needed to assess the power of MACS technique in assisted reproduction.

On the other hand, development of clinical grade products, manufactured according to good manufacturing practices (GMP), which is absent in the market today, will be useful to carry the MACS technique from research to clinical use in assisted reproduction and will also help to develop the product quality in terms of both clinical results and regulatory status of certain countries.

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Polscope-Based Sperm Selection

30

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Abstract

In a time in which the severe male factor affects at least 50% of infertile couples and ICSI is becoming more and more necessary, refining the selection criteria that help identifying the fertilizing spermatozoon might substantially contribute to the establishment of a healthy pregnancy. The properties of birefringence in the sperm head seem to represent a novel approach aimed at maximizing the viability of the resulting embryos. The clinical data reported are promising and support further application of this strategy.

Keywords

Birefringence • Oligoasthenoteratospermia • Azoospermia • Male factor infertility • Non-picnotic sperm • Polscope • Birefringent spermatozoa • Aneuploidy

The Sperm Cell

The viability of an embryo is strictly related to the competence of the corresponding gametes, and for this reason, an increasing interest in the criteria guiding gamete selection has arisen. The final aim consists in identifying the most viable embryos to increase the chances of implantation with the concomitant reduction in the number of embryos to be either transferred or cryopreserved. Although the oocyte has a preponderant role in determining embryo viability, the identification of the fertilizing sperm for ICSI is of comparable importance, especially in those conditions in which sperm motility and morphology are severely compromised.

The techniques available for the analysis of sperm cells have evidenced that a link exists between the quality of sperm and male infertility with an inverse correlation between sperm indices and the occurrence of numerical and structural chromosome abnormalities that are more frequent in infertile men when compared to the normal population [1, 2].

The highest incidence of abnormalities is detected in patients with severe oligoasthenoteratospermia (OAT) and azoospermia, and in patients with high levels of FSH. In these cases, the high proportion of chromosomally abnormal spermatozoa could result in an increased reproductive risk due to the production of embryos with aneuploidy of male origin [3–5].

It is well known that the risk of aneuploid conceptions is mainly associated with maternal age, the contribution of the male gamete being related to a limited number of cases [6]. Nevertheless, ICSI has given the possibility of reproduction to patients that otherwise were destined by nature to remain childless, and it cannot be excluded that the recognized maternal prevalence to the generation of aneuploid fetuses in natural conceptions could be reversed in cases of severe male factor infertility, including azoospermia.

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Moreover, as the sperm centrosome is responsible for the organization of the mitotic plates at the first oocyte divisions, the presence of centriolar defects can cause failed cleavage or abnormal cleavage with an abnormal distribution of chromosomes to the resulting blastomeres [7]. Ultra-structural studies have demonstrated that dysfunctional centrioles are present in sperm with altered motility, and the resulting embryos could be highly abnormal [8, 9].

In light of these findings, it is clear that the quality of the sperm sample has an effect not only on the fertilization and implantation rates after ICSI but also on the incidence of abnormalities in the predisposition to chromosomal errors that increase proportionally to the severity of the male factor condition [5, 10–13].

To further improve the sperm selection procedure during ICSI, novel strategies have been proposed that could assist the embryologist in the identification of the most viable spermatozoa without affecting their vitality. One of these techniques is based on the properties of birefringence of sperm cells which permits a meticulous analysis of sperm morphology in vivo, having no adverse effect on sperm motility or on membrane integrity.

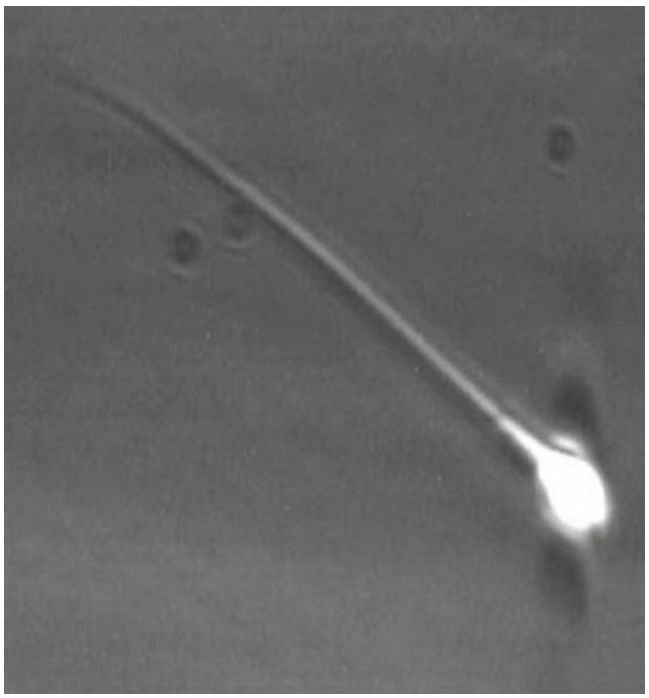

Fig. 30.1 Presence of birefringence in a spermatozoon as observed at the polscope. The birefringence is localized in the acrosomal region, in the nucleus and in the midpiece

Properties of Birefringence in the Sperm Head

Birefringence, or double refraction, is the decomposition of a ray of light into two rays travelling at different velocity (the emerging fast ray and the emerging slow ray) when it passes through an anisotropic material depending on the polarization of the light. The retardance of the slow ray relative to the fast ray generates the birefringence effect.

The sperm cells of many different species, including the human, are naturally birefringent due to the organization of their protoplasmic texture and more specifically the nucleoprotein filaments and the subacrosomal protein filaments which are ordered in rods and longitudinally oriented in the nucleus and in the acrosome, respectively. The same is true for large portions of midpiece and tail (Fig. 30.1).

Comparative studies performed by transmission electron microscopy (TEM) confirm that the presence of birefringence in the sperm head characterizes non-picnotic sperm nuclei and normal acrosomes. Considering that the information provided by the polscope is closer to that derived by TEM than by the conventional phase contrast microscopy, a normal pattern of birefringence is considered to reflect the good health of a sperm cell [14]. Accordingly, it has been found that the fraction of birefringent spermatozoa varies proportionally to the sample concentration, vitality and motility (Fig. 30.2) and that the proportion of birefringent sperm decreases significantly in severe OAT samples and in testicular spermatozoa retrieved by testicular sperm extraction (TESE) compared to normospermic samples (Fig. 30.3).

Based on these findings, it has been proposed to perform ICSI in a microscope equipped with a polarizing lens, or polscope, to select sperm cells having an intact organellar organization as reflected by their birefringence appearance.

Polscope-Based Sperm Selection During ICSI

To test the clinical validity of this approach, a prospective randomized study has been designed including 112 sperm samples from patients undergoing ICSI in which sperm were selected for injection on the basis of their birefringent properties (study group). The laboratory and clinical results were compared with those derived from 119 patients undergoing conventional ICSI (control group). All included cases presented with various sperm sample characteristics: normospermia, OAT with progressive motility, OAT without progressive motility and TESE, which were equally represented in the study and control group [15]. While fertilization and cleavage rates did not differ between the two groups, the ongoing pregnancy rate was significantly higher in the study group (23%) when compared to the controls (11%; $P<0.025$) due to a higher incidence of abortions in the controls (41 vs. 16% in the study groups, $P=0.035$). When analysed according to type of sperm sample, no significant differences appeared in the treatment of normospermic patients and in OAT cases having spermatozoa with progressive motility.

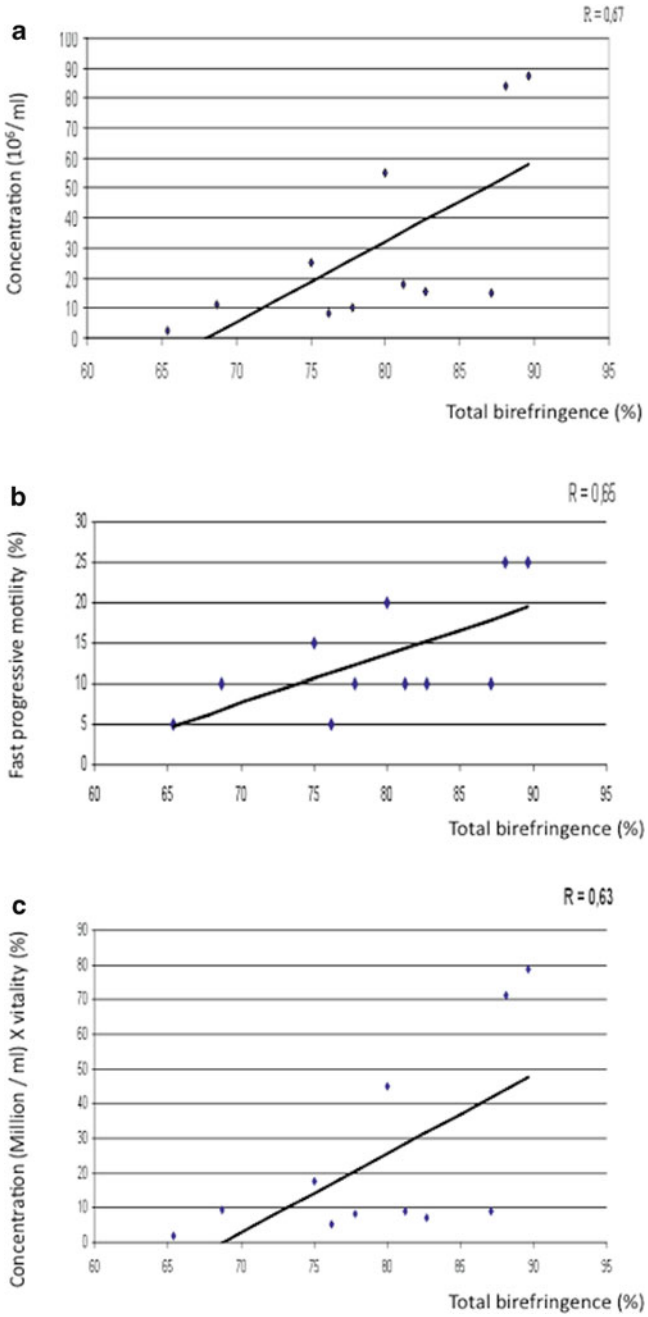

Fig. 30.2 Correlation between the proportion of birefringent spermatozoa and sperm sample parameters. The correlation was positive for sperm concentration (**a**), progressive motility (**b**) and vitality (**c**)

Conversely, the ongoing clinical pregnancy rates and implantation rates were significantly increased in the study group compared to the controls in the most severe male factor condition (OAT with no progressive motility and TESE) (Fig. 30.4).

The conclusions of this study support the properties of birefringence as an important criterion for the selection of spermatozoa to be injected. This strategy could represent an accurate and novel method for an improved clinical outcome in patients with extremely severe male factor infertility and azoospermia for which testicular spermatozoa were used.

The use of the polscope has offered also the possibility, confirmed by TEM, to distinguish between spermatozoa that already underwent the acrosome reaction (Fig. 30.5) from those in which the acrosome is still intact (Fig. 30.1). According to a prospective randomization including 71 couples with severe male factor infertility, the polscope was used to select spermatozoa for ICSI based on the pattern of head birefringence. In all, reacted spermatozoa were injected into the oocytes from 23 patients, non-reacted spermatozoa were injected into 26 patients' oocytes, while in 22 patients, both reacted and non-reacted spermatozoa were injected [16]. There was no apparent effect on the fertilization and cleavage rates of either type of sperm, but a higher implantation rate resulted in the group of oocytes injected with reacted spermatozoa (39.0%) vs. those injected with non-reacted spermatozoa (8.6%, $P = 0.002$). In the group of patients in which the type of injected spermatozoa was mixed, the implantation rate (24.4%) was still superior to that detected in the group of non-reacted spermatozoa (8.6%, $P = 0.048$). Most importantly, the delivery rate per oocyte pick-up followed the same trend suggesting that the injection of reacted spermatozoa seems to have higher chances of generating viable embryos. Similar results have been confirmed by others [17].

Birefringence Analysis at High Magnification

In order to evaluate in more detail the morphology of sperm cells during ICSI, the polscope (Leica DMIRB, Leica Microsystem, Wetzlar, Germany) has been equipped with a PL Fluotar L63X objective, and the images are enhanced by digital imaging (Leica Microsystem). The use of the zoom connected to the camera provides a total magnification on the monitor screen between 2,500 and 5,500× permitting to analyse the characteristics of birefringence in single spermatozoa by observing the corresponding images in real time on the computer screen. In addition, a dedicated software allows to measure sperm dimensions and particularly vacuolar area, providing an accurate classification of sperm morphology in fresh samples assisted by enhanced magnification (Fig. 30.6). In this way, sperm cells can be selected at the same time on the basis of their morphology and birefringence patterns. A recent study has revealed that 7% of motile spermatozoa with normal morphology possess abnormalities in their protoplasmic structure as revealed by the absence of head birefringence. This proportion increases significantly in morphologically abnormal spermatozoa and even more in immotile spermatozoa, where it is close to 40% [18].

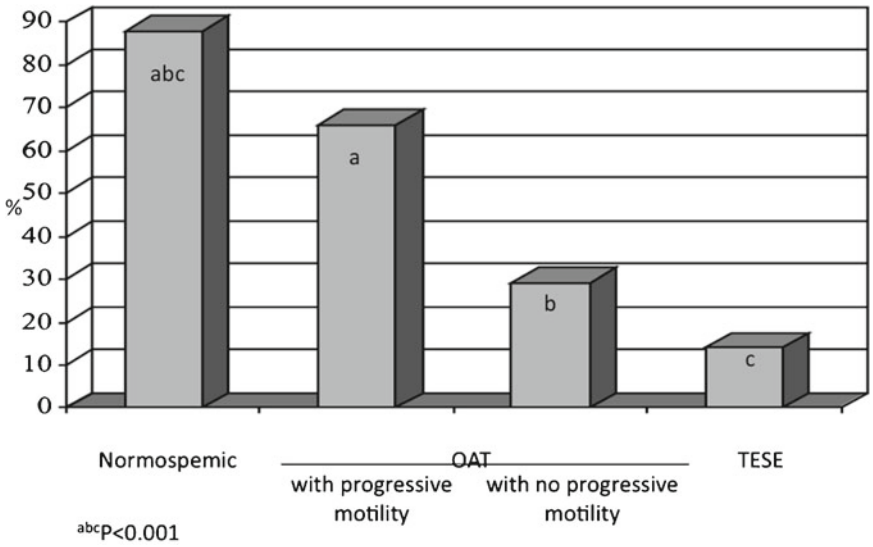

Fig. 30.3 Proportion of spermatozoa with birefringent head in the different sperm types

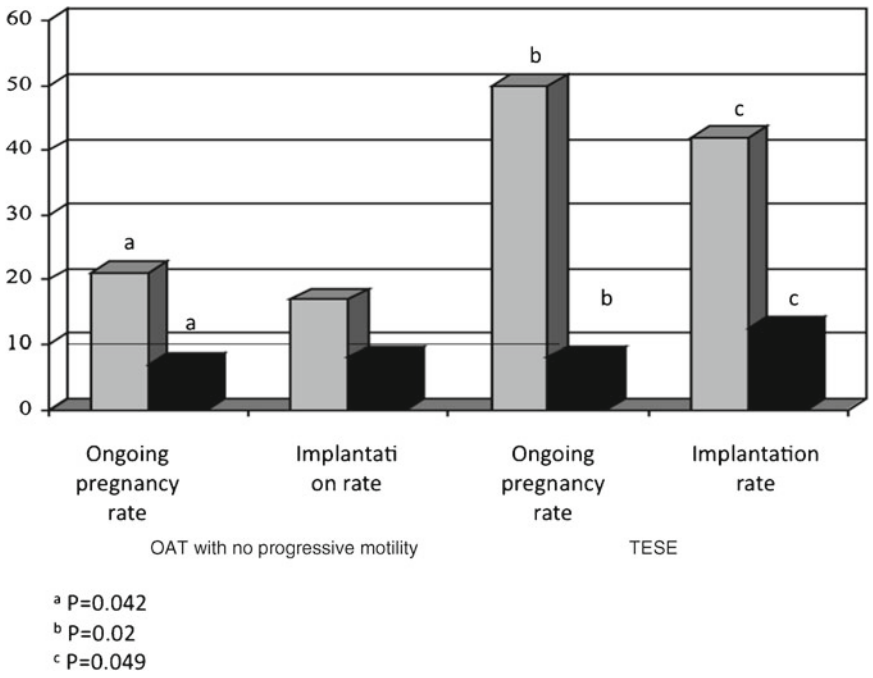

Fig. 30.4 Ongoing pregnancy rate per cycle and implantation rate in OAT patients with no progressive motility and in TESE patients. The study group corresponds to *grey bars* and the control group to *black bars*

Birefringence, Aneuploidy and DNA Integrity

Combining the study of sperm birefringence properties with the assessment of aneuploidy in the same sample, an inverse correlation was observed between the frequency of aneuploidy and the proportion of birefringent spermatozoa [19]. As a result, the selection of birefringent spermatozoa increases the chances of identifying a sperm cell having a normal chromosomal complement. Furthermore, it was found that the proportion of sperm head birefringent spermatozoa is inversely correlated to the incidence of fragmented DNA, suggesting that the selection of birefringent spermatozoa also increases the chances of identifying a vital sperm cell having an intact DNA strand [20]. Therefore, performing ICSI under a polarizing lens minimizes the risk of selecting spermatozoa having DNA fragmentation as well as an aneuploid condition. These observations could be another reason for the favourable clinical outcome associated to the use of the polscope for sperm selection in severe cases of male infertility.

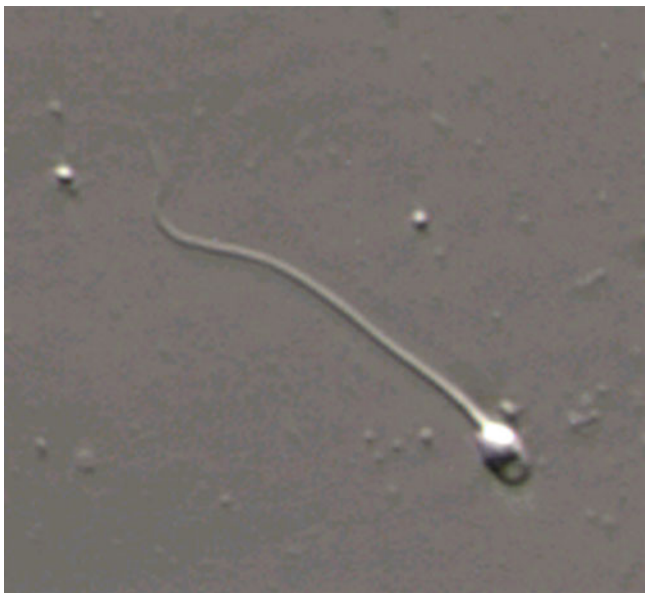

Fig. 30.5 The localization of birefringence in the postacrosomal region indicates that the acrosome is already reacted

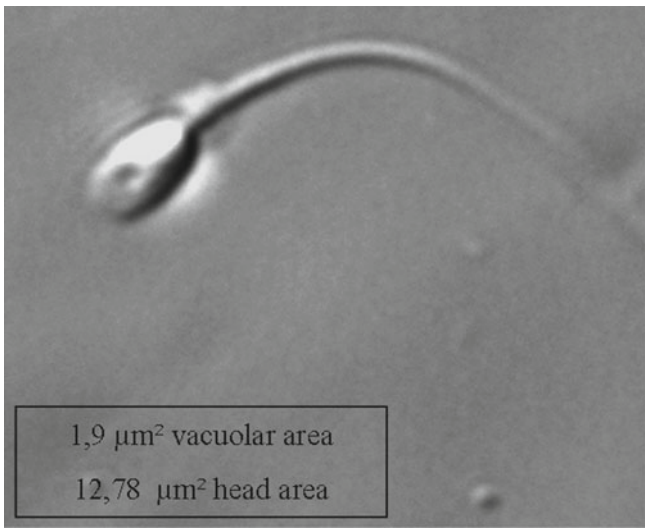

Fig. 30.6 The software allows to measure sperm dimensions and vacuolar area. Therefore, birefringence characteristics and morphology at high magnification are evaluated at the same time

Conclusions

In a time in which the severe male factor affects at least 50% of infertile couples and ICSI is becoming more and more necessary, refining the selection criteria that help identifying the fertilizing spermatozoon might substantially contribute to the establishment of a healthy pregnancy. The properties of birefringence in the sperm head seem to represent a novel approach aimed at maximizing the viability of the resulting embryos. The clinical data reported are promising and support further application of this strategy.

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Part VI

Insemination Procedures

Intrauterine Insemination

31

Gautam N. Allahbadia and Rubina Merchant

Abstract

Intrauterine insemination (IUI) is an assisted reproductive technique that involves the deposition of a processed semen sample in the uterine cavity, overcoming natural barriers to sperm ascent in the female reproductive tract. It is a cost-effective, noninvasive first-line therapy for selected patients with functionally normal tubes, and infertility due to a cervical factor, anovulation, moderate male factor, unexplained factors, immunological factor, and ejaculatory disorders with clinical pregnancy rates per cycle ranging from 10% to 20%. It, however, has limited use in patients with endometriosis, severe male factor infertility, tubal factor infertility, and advanced maternal age ≥35 years. IUI may be performed with or without ovarian stimulation. Controlled ovarian stimulation, particularly with low-dose gonadotropins, with IUI offers significant benefit in terms of pregnancy outcomes compared with natural cycle or timed intercourse, while reducing associated COH complications such as multiple pregnancies and ovarian hyperstimulation syndrome (OHSS). Important prognostic indicators of success with IUI include patient's age, duration of infertility, stimulation protocol, infertility etiology, number of cycles, timing of insemination, number of preovulatory follicles on the day of hCG, processed total motile sperm >10 million, and insemination count $>1 \times 10^6$ with >4% normal spermatozoa. Alternative insemination techniques, such as Fallopian tube sperm perfusion, intracervical insemination, and intratubal insemination, provide no additional benefit compared to IUI. A complete couple workup that includes patient history, physical examination, and clinical and laboratory investigations is mandatory to justify the choice in favor of IUI and guide alternative patient management, while individualizing the treatment protocol according to the patient characteristics with a strict cancelation policy to limit multifollicular development may help optimize IUI pregnancy outcomes.

Keywords

Intrauterine insemination • Assisted reproduction • Ovarian stimulation in intrauterine insemination • Fallopian tube sperm perfusion • Intracervical insemination • Cervical factor infertility

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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, noninvasive, 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. 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 preprocedure 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.

Indications for IUI

Cervical Factor Infertility

In couples with a cervical factor, diagnosed by a well-timed, nonprogressive postcoital 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].

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].

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].

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 to 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].

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 the 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 three 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 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 3–4 cycles [19].

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].

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 laparohysteroscopy 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), 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 trachomatis, *Chlamydia trachomatis*, and *Neisseria gonorrhea*.
- Testing for HIV/hepatitis.

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, sperm fertilizing ability.
- Testing for antisperm antibodies.
- Semen culture and sensitivity to evaluate infection.
- Testing for HIV/hepatitis.

Natural Cycles or Controlled Ovarian Hyperstimulation?

IUI may be performed with or without controlled ovarian hyperstimulation depending on the patient characteristics.

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 31.1), while no significant differences were observed between natural cycles and donor insemination 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 cycles 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].

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.

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

Table 31.1 Comparison in the clinical pregnancy rates following stimulation with various regimens and IUI

		Stimulation regimens							
			CC + gonadotropins			Aromatase inhibitors		Gonadotropins		
Study	Natural cycles	CC	hMG	rFSH	Tx + G	Letrozole	L + rFSH	hMG	rFSH	*P* value
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

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 rFSH, a randomized multicenter parallel trial concluded that being less expensive, CC seems the more cost-effective drug and therefore, can be offered as drug of first choice [32].

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 3,957 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 protocols 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.

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 31.1. 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 ($P < 0.001$) rate compared to CC (100 mg/day for 5 days) and despite a higher gonadotropin dose and stimulation duration and fewer follicles larger than 14 mm than clomiphene-treated patients [37].

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 31.1). 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].

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).

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.

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 [46].

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.

The Short GnRH Antagonist Protocol

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.

Incremental Dose Protocol

Following the initiation of gonadotropins on day 3 of the menstrual cycle, antagonists, cetrorelix/ganirelix, are administered in an incremental dose (0.25 mg/day when the leading follicles are 14 mm and increased to 0.5 mg when the leading follicles are 16 mm) until hCG administration.

The Long GnRH Analogue 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].

Ultralong Agonist Protocol

The ultralong 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 [47]. However, the efficacy of the antagonist in preventing premature luteinization is disputed with some studies reporting in favor [48] and others against [49, 50].

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 [51, 52].

Semen Parameters and Processing

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 [53]. 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) <10 million [54] sperm survival <70% [55], <5% normal spermatozoa, inseminating motile count (IMC) $<1 \times 10^6$ [7], and prewash IUI-semen pregnancy score (IUI-SPS) <150 [56], necessitating alternative therapy. The PTMS count has been independently associated with fertility after IUI ($P=0.0014$) [54]. PTMS $\geq 10 \times 10^6$, their 24-h sperm survival threshold of ≥70% [55], 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 [57]. These variables would be helpful in counseling patients for future management [57].

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 [58].

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, SilSelect, 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 [59] while the Cell Sorter-10 (CS-10) preferentially isolates spermatozoa with very low levels of DNA damage. Noncentrifugation methods include a novel Zech device that is a dual-chamber capillary dish that obviates the potential harmful effects of centrifugation on sperm.

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 nonapoptotic spermatozoa, and annexin V molecular glass wool filtration [60].

Density Gradient Centrifugation versus Swim-Up/Sperm Wash

DGC techniques, utilizing PureSperm, yield significantly higher median total motile sperm counts (TMSC) [32.2×10^6 vs. 17.6×10^6], higher recovery rates of mature motile sperm (69.2% vs. 50.0%), longevity at 4 h (83.0% vs. 55.0%), postthaw recovery, and better preserve semen quality in fresh and cryopreserved semen compared to swim-up [61]. 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) [62] and significant sperm enrichment compared to Isolate

(50% and 90%) [sperm recovery rates (30.0% vs. 19.7%, respectively)] [61]. 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 [63]. 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 [62]. 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 [58].

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 [65], significant improvement in induced acrosome reaction test (IART) ($P<0.001$) [66] with an improved ability to fertilize eggs using the hamster oocyte penetration assay [60], and significantly reduced levels of apoptotic markers versus DGC alone ($P<0.001$) [66]. 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.

IUI in Practice: Description of the Procedure

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 (2,623 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 [67]. Despite comparable pregnancy rates between hCG-induced ovulation and LH surge (an increase in LH level ≥200% over a mean of preceding 2 days) timed IUI, a significantly longer time to IUI, significantly higher cancelation rates (31% vs. 11%) attributed mainly to failure to detect an LH surge [68], and favorable outcomes limited only to CC-induced cycles [69] have been reported suggesting that the decision to use hCG for IUI timing should be influenced by factors other than pregnancy rates [68]. 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 [70].

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 [71].

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 volsellum, 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 [72].

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 heart beats 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 per patient (21.1% and 39.4%, respectively) compared to patients who received no luteal phase support (12.7% and 23.8%, respectively) [73]. Alternatively, intranasal administration of a GnRH agonist (buserelin) could be effective in providing luteal support with good pregnancy rates (28%) [74].

Postprocedure 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.

Single vs. 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, 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 [75] and slightly higher cost of double IUI [76]. 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 [77]. 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 that the 24th-h IUI might be preferred in demanding situations [78].

IUI vs. 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 [79], a recent meta-analysis [80] failed to demonstrate a significant clinical benefit in terms of pregnancy rates between stimulated and natural cycle IUI, natural cycle TI/IUI, or 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 [80]. 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 [81].

Number of Cycles

There is general agreement in the literature that 4–6 IUI cycles may be performed with acceptable pregnancy rates before resorting to alternative therapy [62, 82] 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) or six (CPRs 65% for ovulation dysfunction, 35% for endometriosis, and unchanged for other diagnoses) cycles [82]. Morshedi et al. [62] 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% per with a miscarriage rate of 34.0% [62]. Aboulghar et al. [83] reported significantly higher cycle fecundity in the first three trials of COH-IUI than in cycles 4–6 (16.4% vs. 5.6%, $P<0.001$, $n=594$, 1,112 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 [83]. Moreover Qublan et al. [84] reported an increased incidence and recurrence rate of luteinized unruptured follicle (LUF) among patients with preexisting LUF undergoing multiple IUI cycles. The incidence of LUF was 56.5% in the second cycle of IUI treatment with 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 [84]. 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 [85].

Intrauterine versus Other Forms of Artificial Insemination

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 [86] and the consequent risk of infection and

anaphylaxis [87]. 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 [86].

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 [88]. 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, reduced cost and complications in couples with unexplained infertility compared to IUI [89, 90], 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 [88]. In couples with longstanding infertility, intraperitoneal insemination (IPI), FSP, and IUI have similar efficacy in the achievement of a clinical pregnancy [91].

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 [92].

Risks and Complications of IUI

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 [93]. 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 [94]. 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 [95]. Infectious complications associated with IUI are frequently cited, though rarely reported. According to Sacks and Simon [96], 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 [97]. *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 [97].

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, 80]. Miscarriage rates ranging from 11.8% [6] to 34.0% [4] have been reported following COH-IUI.

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 [98], 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 [99]. 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 [100, 101]. According to van Rumste et al. [100], 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 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 and 2.8% after multifollicular growth [100]. 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 >1,000 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 >1,000 pg mL, whereas three or more implantations were not significantly increased [101].

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) [102]. 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 [103]. 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. [104] 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 [104].

Success Rates with IUI

Factors Affecting Outcome

Overall CPRs/cycle ranging from 9.2% to 22% have been reported in the literature following stimulated IUI [4, 6, 7, 79, 96, 120] with live birth rates of 10% for CC-stimulated and 8.7% for rFSH-stimulated cycles [37]. Guven et al. [105] 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 [106].

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 [62]. 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 [107].
- 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 [108]. Cervical factor yields a favorable outcome [109].
- Sperm parameters
  - Total motile sperm count (TMSC) before sperm preparation ($P>0.05$) [105].
  - 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% [110]
  - Progressive motility [99].
  - Normal sperm morphology [105, 106]. 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; $p=0.003$) [105].
  - Processed total motile sperm count (PTMS) count $\geq 10 \times 10^6$ and 24-h survival of ≥70% can predict the IUI outcome with a 94% sensitivity and an 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 [111].
- Mild controlled ovarian hyperstimulation [107, 109, 112]. 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 [112].
- Follicular dynamics.
  - Increase in the number of preovulatory follicles (1–4) at the time of hCG administration [2, 99, 109, 113].
  - Increase in AFC and, hence, increase in the dominant follicle number and E2 level on the day of hCG [7].
  - Serum E2 levels. Birth rate increased from 3.6% when E2 was <500 pg/mL to 19.6% when E2 was ≥2,500 pg/mL [109].
  - 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 [112]. However, a recent study by Ragni et al. [114] 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 [114].
- Endometrial thickness [99].
- Shorter duration of infertility [99].
- Cervical mucus aspiration before IUI [60].

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% following CC stimulation in endometriosis-based infertility, and 24.3% with an IMC $>30 \times 10^6$. Ovarian stimulation in unexplained infertility resulted in PRs of 24.2% and 19.8% with three follicles and an IMC $>30 \times 10^6$, respectively [98].

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 [107–109]. 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 [115]. 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 [116].
- 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 [117].
- Male factor infertility with initial sperm motility <30% ($P < 0.002$), initial sperm count $<5 \times 10^6$ per mL ($P < 0.01$) [114], and normal sperm morphology <14% [112].
- Postwash TMSC ≤20 million/mL [108].
- Endometriosis and tubal factor-associated infertility [106–109].
- Uterine anomalies [109].
- Increasing number of cycles with more than four treatment cycles [107, 109].
- Longer duration of subfertility [109].
- Low midluteal progesterone (P4) level ≤25 nmol/L [118].

A recent study by Merviel et al. [119] 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 ≥5 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 [119]. 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 [106].

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 [120]. 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 [121].

Conclusion

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 $\geq 10 \times 10^6$ and a 24-h survival of ≥70%, normal sperm morphology of >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, 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.

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Conventional IVF Insemination

32

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Abstract

Our objective with this chapter is to give a brief overview of the conventional in vitro fertilization (IVF) procedure and some of the factors that determine success. The decision-making process of whether to do conventional IVF, intracytoplasmic sperm injection (ICSI), or split-ICSI is very complex. We present the influencing factors in a manner that allows thorough evaluation of every case, resulting in an educated, well thought through decision of which insemination method to use. We believe it will assist embryologists in deciding which method of fertilization to use in their labs.

Keywords

Conventional in vitro fertilization • Intracytoplasmic sperm injection • Comparison of IVF and ICSI • History of IVF • Split-ICSI • IVF procedure

As with most other medical practices, infertility specialists customize patient treatment plans according to the need and characteristics of each individual case. This is especially true for ovarian stimulation and synchronization of the patient's cycle prior to oocyte retrieval, where the physician makes decisions based on several different variables along the course of the treatment. Once oocytes have been obtained, this decision-making process continues in the laboratory.

Often, the first of such choices that embryologists are faced with is which method of insemination to use. Conventional in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are distinct in methodology, and deciding which of these routes to follow can be quite complex. Numerous factors play a role in the appropriateness and outcome of each method. Furthermore, embryologists may choose to split the oocytes into two different groups and perform ICSI on one group and IVF on the other group. In such cases, the treatment is referred to as split IVF/ICSI.

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This section is aimed at describing the method and some important elements of conventional IVF (from here on, IVF refers to conventional IVF). We will also compare the advantages and disadvantages of the three options and discuss the different factors that should be taken into consideration when deciding which insemination method to use. Figure 32.1 was compiled in attempt to aid the decision-making process. We hope this will help embryologists, physicians, and patients to make the most appropriate and informed choices.

History of In Vitro Fertilization

The first report of IVF came from Spallanzani in the 1700s, when he demonstrated the fertilization of frog oocytes by mixing them with semen (for review, see ref. [1]). Although he had previously seen spermatozoa using his various microscopes, at the time, it was believed that spermatozoa were parasites instead of the fertilizing agent. Only in the next century, with much opposition, were Prevost and Dumas able to demonstrate that spermatozoa are *secreted* by the testes and responsible for the fertilizing capability of semen [1]. This was followed by von Baer's discovery of the mammalian oocyte in 1827 and eventually successful IVF and births

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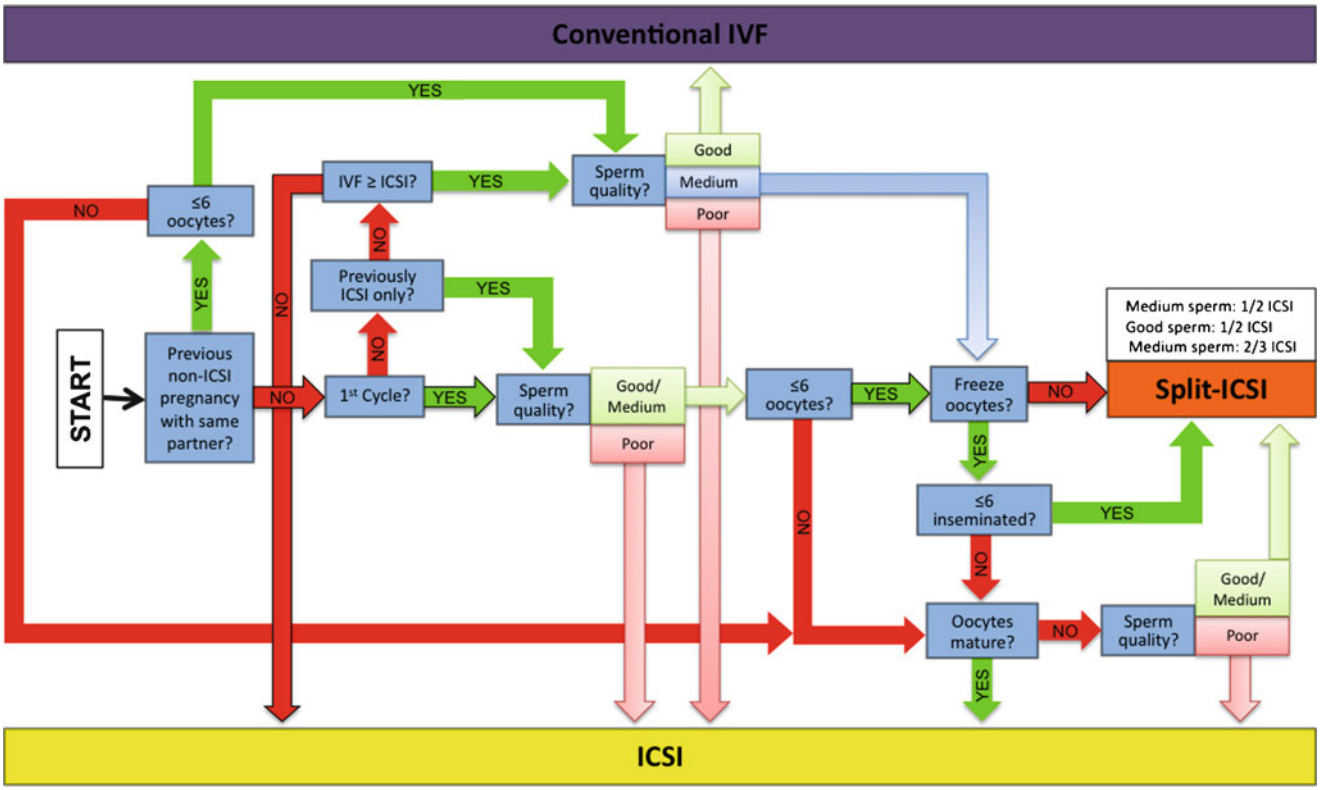

Fig. 32.1 Flow chart for decision-making in insemination choices

in the rabbit by Chang [2]. Realizing the clinical value of this discovery, several groups in Europe, Australia, and the USA competed to achieve successful IVF in humans. On 25 July 1978, Louise Brown, the first human birth resulting from IVF, came from Steptoe and Edwards in the UK [3]. More live births followed shortly in Australia (1980), the USA (1981), and Sweden, France, and Austria (1982), with a subsequent worldwide explosion of ART clinics and research programs (for review, see refs. [1, 4–6]). One debated topic was the use of stimulated vs. natural cycles, as Louise Brown was a result of a natural cycle after hormonal stimulation was abandoned by Steptoe and Edwards due to repeated failure [3]. Eventually, successful stimulation protocols that allowed multiple oocyte collection were developed [7]. This allowed production of multiple embryos from a single cycle, embryo selection, and the ability to cryopreserve the extras. Furthermore, having numerous oocytes opened up the possibility to use more than one method of insemination in a single cycle. We will elaborate on this topic in subsequent sections.

Initially, IVF was the only available option for creating *test-tube babies*. This meant that for successful fertilization, spermatozoa that were placed in large numbers in the same tube (dish) as oocytes had to find an oocyte, infiltrate the cumulus, bind to and penetrate the zona pellucida, attach to the oolemma, and finally deposit its nuclear material in the ooplasma, all under in vitro conditions [3, 8]. It also meant that during IVF, many of the natural selection processes were still in place. For example, to successfully fertilize the oocyte, the spermatozoan had to, among other factors, be progressively motile to enter the cumulus mass and be able to undergo the acrosome reaction to penetrate the zona [8]. It was soon realized that for certain cases, some of these events had to be circumvented in order to obtain fertilization. This was particularly true for patients with male-factor infertility that would not normally qualify for IVF due to abnormal morphology, low motility, and low sperm concentration [9, 10]. Therefore, experimentation for alternative modes of fertilization was underway. Initially, studies focused on assisting spermatozoa microsurgically to get through the zona pellucida. Methods included making an opening for sperm to enter through the zona either mechanically (i.e., partial zona dissection) or chemically (zona drilling) [11], or by injecting the sperm into the perivitelline space (subzonal sperm injection) [12]. Although these techniques increased fertilization rates, they had drawbacks, such as increased rates of polyspermy and the fact that the sperm still needed the capacity to bind to the oolemma [13]. Eventually, a procedure known to be successful in rabbits [14] and cattle [15], in which the spermatozoa were injected directly into the

ooplasma, led to the first human pregnancies and subsequent birth from ICSI [16].

Development of ICSI gave embryologist the *luxury* of a choice between two different approaches, IVF or ICSI, each with unique advantages and disadvantages, which will be discussed below. The ability to choose, however, is only beneficial if factors that determine success rates for each method are carefully considered and understood.

Elements of Conventional IVF

In one sentence, conventional IVF can be described as follows: Obtain oocytes from a natural or stimulated cycle, obtain sperm either surgically or from ejaculate, mix the gametes in a dish with appropriate culture medium, and culture overnight. The next day, fertilization is accessed by the presence of male and female pronuclei. Although this may sound pretty straight forward, there are dozens of elements throughout the procedure that have been fine-tuned over more than 30 years to obtain the success we see today. The most basic ones include factors involving gamete collection, processing, and subsequent embryo culture. Previous chapters have described these techniques in detail. Therefore, this section will only give a brief overview of the insemination procedure and then focus on some aspects that have not been discussed.

The In Vitro Insemination Procedure

Different programs use different protocols for IVF. This brief description corresponds to the methods that are utilized at our practice, but are not necessarily superior to differing protocols.

Gametes are collected and processed as previously described. Oocyte maturity is assessed at time of retrieval, based on cumulus maturation [17, 18]. Although oocytes that appear immature can still be inseminated, the embryologist should keep record of the number of immature oocytes. This will help with more accurately evaluating fertilization outcomes.

Prior to insemination, it is of utmost importance to cross-check patient sample identification numbers to make sure that the correct oocytes are inseminated with the correct sperm. Needless to say, error at this step can have dire consequences. A carefully labeled Petri dish with drops of fertilization medium under mineral oil can be prepared the night before to ensure thorough equilibration in the incubator overnight.

Insemination normally takes place roughly 5 h after oocyte retrieval and 40–41 h after hCG administration. Processed sperm is added to the insemination drop to obtain a final concentration of approximately 1×10^5 cells per mL. For a cell suspension of 10×10^6 per mL, the addition of 10 μL sperm preparation to an insemination drop of 90 μL should provide the appropriate concentration. Oocyte-cumulus complexes (OCCs) are then added to the fertilization drops. We typically add no more than 4 OCCs to a drop of roughly 100 μL (~25 μL of medium per OCC). Gametes are cultured overnight in a humidified incubator, usually at 5% CO_2 (depending on medium formulation) in *fertilization medium*.

The next day, oocytes are washed, manually stripped of cumulus (the details of the stripping procedure is described in Chap. 12), and transferred to fresh drops of *cleavage medium*. Fertilization is assessed by the presence of two pronuclei, and subsequent embryo evaluation is performed as described.

Timing of Insemination

To achieve successful fertilization, oocytes need to be fully matured in preparation for interaction with sperm upon fertilization. This includes nuclear and cytoplasmic maturation of the oocyte, expansion of the cumulus and corona, preparation of the zona pellucida and oolemma for sperm binding, and extrusion of the first polar body. The result is a fully matured oocyte arrested in metaphase II, ready for the complex cascade of sperm-oocyte interactions that will follow (for recent reviews, see refs. [8, 19, 20]). In a natural cycle, these events are timed by the gonadotropins synthesized in the anterior pituitary. They are controlled by gonadotropin-releasing hormone (GnRH) from the hypothalamus and ensure completion of oocyte maturation prior to sperm exposure and fertilization. Follicle-stimulating hormone (FSH) initiates follicular recruitment of FSH receptor-bearing, recruitable follicles, which leads to growth of the cohort and eventually maturation of the dominant follicle. At the end of the follicular phase, due to a positive feedback by estrogen on the pituitary, the LH surge occurs and triggers ovulation. A delicate relationship is maintained between the oocyte and follicular cells so that maturation of both is carefully synchronized, leading to ovulation only once the oocyte has fully matured [21]. The oocyte is released into the peritoneal cavity, captured by the fimbria, and transported to the oviductal ampulla, where fertilization typically occurs [8, 22]. Therefore, after ovulation, an unspecified window (minutes to hours) of time elapses between ovulation and fertilization, during which final oocyte maturation processes can be completed.

In a stimulated cycle, the woman is treated with high doses of gonadotropins to ensure that numerous follicles will mature while ovulation is suppressed (for review, see ref. [23, 24]). Maturation and ovulation is then initiated by hCG treatment, followed by oocyte aspiration (instead of ovulation) 35–36 h later, mimicking the in vivo LH-to-ovulation

time frame, but not necessarily allowing completion of maturation. It should also be noted that should insemination be performed immediately following oocyte retrieval, the time of capture and transport to the ampulla is essentially lost, implying that a significant percentage of oocytes may not have had the opportunity to fully mature.

There appears to be asynchrony between the somatic cells and the oocyte in stimulated cycles, which leads to lower fertilization rates when insemination is performed immediately after oocyte retrieval [25]. In fact, Trounson et al. [26] reported the highest fertilization and pregnancy rates after a 5–6-h delay of insemination following oocyte retrieval 34–37-h post-hCG. They also found that fertilization rates dramatically decreased as the timing of insemination after oocyte retrievals decreased. Considering these results and the mentioned asynchrony in the OCC, it is not surprising that Cohen et al. found that after hCG stimulation, the percentage of visible meiotic spindles increased with time elapsed after hCG injection, even in oocytes that already extruded the first polar body [27]. As a result, the authors suggested postponing ICSI to 38–42 h after hCG administration, which apparently compensates for some of the asynchrony and lost migration time.

These and other reports indicate that timing of insemination in relation to hCG administration is imperative to achieve oocyte maturation and successful fertilization. It also appears that the timing of insemination after oocyte collection is less critical since the last phases of the maturation process is initiated by hCG administration and not by removal of the oocyte from the follicles. Needless to say, follicles should have reached a minimum level of maturity (usually 1.4 cm in diameter) before hCG and aspiration is attempted to make sure the OCC is receptive for stimulation.

Nuclear maturation can occur outside of the follicle, providing that the minimum threshold of maturation has been reached (i.e., fully grown germinal vesicle stage). However, cytoplasmic maturation is also essential for fertilization, which is supported by the somatic cells [21]. The OCCs therefore has the ability to continue the maturation process in vitro and may potentially be fertilized hours after their sibling oocytes, as long as they are given enough time and have the support of the maturation-promoting cumulus [28]. We will discuss handling of these immature OCCs in subsequent sections, but in general, recommend that oocytes should be inseminating no less than 38 h after hCG.

Concentration of Spermatozoa During Insemination

The main objective of IVF is to achieve fertilization outside of the body. As the procedure became more common, so did the challenges that embryologists faced. The first measure of success that is evaluated in the laboratory is fertilization, as indicated by the presence of two pronuclei and the second polar body the day after insemination (following mechanical removal of the cumulus, the procedure that is described in detail in Chap. 12). Failed fertilization was one such a problem that had to be faced early on. Researchers started trying different procedures in attempt to increase the chances of fertilization, which led to development of several new techniques, including ICSI. One such method that received significant attention in the late 1980s and 1990s was to increase the sperm concentration for patients with sperm morphology abnormalities or those that previously had failed IVF cycles [29, 30]. This was called the high oocyte insemination concentration (HIC) procedure. The idea was that even if there is just a small percentage of motile, morphologically normal spermatozoa in the ejaculate, increasing the insemination concentration would lead to a sufficient number of sperm to fertilize the oocyte.

Literature on the topic is very contradictory. While some groups reported higher fertilization rates with HIC-IVF [29, 31], others found that in couples with total fertilization failure, increased sperm concentration did not rescue fertilization [30]. In patients with severe teratozoospermia, Oehninger et al. [32] obtained higher fertilization rates in oocytes inseminated by HIC than for those on which ICSI was performed. The ICSI group, however, produced embryos with higher morphology scores, and there was no difference in implantation rates between the two treatment groups. The mentioned studies used inconsistent procedures. For example, some denuded oocytes prior to insemination, while others left the OCCs intact. These variations in protocols make results from different publications hard to compare.

At the moment, most research shows superiority of ICSI over conventional IVF to overcome failed fertilization, even when compared with HIC. Therefore, most groups abandoned HIC-IVF in favor of ICSI for couples with a history of failed fertilization following conventional IVF.

Advantages and Disadvantages for Conventional IVF and ICSI

In order to make an informed decision of when to use which procedure, it is important to understand the major advantages and disadvantages of one method over the other.

Conventional IVF is a less invasive, more natural procedure that provides a longer window of opportunity for sperm to fertilize the oocytes, rather than insemination at a specific time point. It maintains several natural selection criteria for sperm as well as oocytes, which may allow for some unknown mechanism to reduce developmental defects. Reports exist of developmental delays in children conceived by ICSI when compared to those from IVF or natural conception [33–36], but whether the lack of such mechanisms in ICSI offspring is responsible for these findings remains to be confirmed.

It is much less labor intensive and cheaper to perform than ICSI, and a large wealth of information is available on the subject since it has been intensively studied in humans for over 30 years. One of the biggest drawbacks of IVF over ICSI is a higher occurrence of failed fertilization [37, 38]. This can be attributed to various causes, such as poor sperm quality, or oocyte defects such as an abnormal zona pellucida or oolemma that renders sperm incapable of fertilizing the oocyte (for recent reviews, see refs. [8, 20]). Therefore, semen quality has to be higher when used for IVF vs. ICSI.

Another disadvantage of IVF is that embryologists generally do not know the maturation status of oocytes, since they are not denuded before insemination. This may skew success rates when comparing the techniques. To minimize this problem, the cumulus maturation aspect can be used as an indication of oocyte maturity [17, 39]. It is also possible to individually examine each OCC in a small volume of media by pulling away the thickest parts of the cumulus to look for the presence of a polar body [18]. This technique, coined *cumulus spreading*, is of course a very time-consuming and tedious procedure.

The most value of the ICSI procedure is that the sperm does not have to bind to or penetrate cumulus and corona, the zona pellucida or the oolemma, as it is injected directly into the ooplasm. Thus, morphologically abnormal or low-motility sperm that may be unable to fertilize oocytes during IVF can be used for injection. It also provides the opportunity to examine each oocyte closely prior to injection, which results in more accurate portrayal of success rates. While denuding adds some advantages, it may affect the oocytes negatively, as hyaluronidase treatment and manual stripping can damage the oocytes. ICSI requires a very high level of skill and precision, which increases the workload of the laboratory exponentially.

As this brief discussion indicated, there are definite advantages and disadvantages to both techniques, and ideally, clinics should find a way to combine their use to reap the benefit of both techniques.

Deciding on IVF, ICSI, or Split-ICSI

Although conventional IVF was the original method of insemination outside of the body, many laboratories have completely switched to ICSI as their preferred method of insemination, regardless of oocyte and semen qualities. There are good arguments for doing strictly ICSI, such as a single, standardized protocol to maintain and less emphasis placed on semen evaluation. However, there are advantages in the ability to perform both techniques, depending on specific case parameters. In doing so, a more customized treatment plan is followed for each patient. Several important factors should be considered when deciding which method, IVF, ICSI, or split-ICSI, to perform. The latter is taking advantages of both methods and thereby essentially *covering all the bases* [38, 40].

It would be very challenging to discuss each issue that determines the most appropriate method of insemination individually. Instead of a simple formula, the decision-making process is very complex, as each aspect is influenced by the others. Furthermore, there are external issues that may alter the preferred route (discussed later). We therefore generated a flow chart that includes the most important factors to consider (Fig. 32.1). These are represented as questions, and the philosophies behind them for the decision-making process are discussed below. Importantly, the chart is by no means all inclusive, but is meant as a guide for physicians and embryologists. There are numerous additional factors that may influence the method of choice, and endless unique situations that may arise and are not on the chart. Such issues were omitted from the flow chart and discussed separately in attempt to simplify it.

Previous Non-ICSI Pregnancy with Same Partner

It is of utmost importance to look at a patient's fertility record if one is to understand the full scope of the treatment options. The question of whether or not a patient previously had a non-ICSI pregnancy gives an indication of former sperm-oocyte interaction. If the answer is yes, it is likely that the sperm has (or at least at some point had) the ability to fertilize the oocytes without injection. The time since the last successful pregnancy (or fertilization using IVF) should be noted, as quality parameters of either gametes may have changed over time. Nonetheless, this history indicates that they are (or at least once were) compatible. If a patient has never conceived without ICSI with her current partner, but both partners previously had proven fertility with different partners, there may be compatibility issues between their gametes regardless of the quality. This may especially affect younger couples with seemingly good-quality semen and oocytes. In such a case, although IVF could be successful, ICSI nevertheless may be necessary to obtain fertilization.

First Cycle

Much information about a patient and the expected outcomes can be gathered from previous cycles. By looking at former cycles, the embryologists should/can get idea of the fertilization capacity of the oocytes and sperm. This is especially true for patients that previously had all IVF or split-ICSI cycles. For example, if a patient responded poorly to IVF but well to ICSI, IVF should not be considered for the next cycle. However, if a patient had better fertilization rates with IVF

than ICSI, the lab may opt to do all IVF or split-ICSI, depending on sperm quality and the number of oocytes available on the day. Therefore, it is essential to thoroughly examine previous cycles and use what was learned from them in subsequent cycles.

In a patient's first cycle, it is especially valuable to do split-ICSI as a means to gather such information for subsequent cycles. The first cycle may even be treated as a test cycle specifically for this purpose and in essence serve as a diagnostic tool. For example, in couples that had fertility with different partners, failure to get pregnant may arise from incompatibility between the sperm and oocytes of that particular couple (see discussion section "Previous Non-ICSI Pregnancy with Same Partner"). In such a case, we would expect high fertilization rates with ICSI and low rates with IVF. To benefit most from such a diagnostic cycle, it should still conform to downstream requirements, as depicted in Fig. 32.1. If the semen quality is poor, doing an ICSI split may not yield accurate information to use in subsequent cycles, and it may be better to treat the cycle as a regular cycle with maximum fertilization the main objective. In that case, according to the flow diagram, only ICSI should be performed.

Sperm Quality

For successful IVF, the sperm needs to have the ability to fertilize the oocyte without assistance. This means that there should be minimum number of morphologically normal, motile sperm in the insemination drop to achieve fertilization (discussed above). If sperm quality is poor and cannot be improved by processing, ICSI should be performed. Good, medium, and poor sperm refer to relative standards, and each sample should be evaluated for specific criteria [41] to estimate whether there would be enough normal, motile sperm from the ejaculate to perform IVF.

Generally, the concentration of sperm in the fertilization drop should be about 100,000–150,000/mL motile, morphologically normal cells. If raw semen quality is too low to meet these criteria, quality should be classified as poor, and all ICSI should be performed to maximize fertilization rates. If the above criteria can be reached but it appears borderline or still have low progressive motility (<40%) or some morphological abnormality, at least 50% of the oocytes should be allocated to ICSI in case IVF fails. Good sperm quality, with sufficient concentration, motility, and morphology, may be used for all IVF or up to 50% IVF in split-ICSI cases. Sperm quality is the final determinant of whether all IVF or split-ICSI will be performed for patient that are known to respond better to IVF than ICSI.

Oocyte Number Recovered from the Patient: If <6 Oocytes

The number of oocytes for this question should be internally standardized for each lab. In general, patients with six or more oocytes should be considered for split-ICSI. More conservative groups may choose a larger number as their cutoff.

During an in vitro cycle, the most critical aim of the lab is to achieve fertilization, since embryos and pregnancies cannot be obtained without it. Therefore, if there is a limited number of oocytes, it becomes more important to focus on the method that will result in higher fertilization rates, which is ICSI [37, 42]. As discussed, conventional IVF has some advantages over ICSI. Therefore, if a patient has more than the specified number of good-quality mature oocytes, a percentage of them can be assigned to IVF. We recommend that split-ICSI should be performed if a patient has a minimum of six mature, morphologically normal oocytes. Each lab, however, should decide their standard cutoff oocyte number for split-ICSI. Typically, oocytes will be divided evenly between the two treatment methods. In some cases, the lab may decide to adjust this ratio based on other parameters, for example, allocate two thirds to ICSI and one third to IVF if sperm quality is medium or below (see Fig. 32.1).

Should We Freeze Excess Oocytes?

Patients with large numbers of oocytes may opt for (partial) oocyte freezing. This will reduce the number of oocytes to inseminate. The number of remaining oocytes left for insemination should thus be considered when determining which method to use. If more than the lab standard remains (typically six or more), split-ICSI should be performed. However, if fewer are left for insemination, the maturity of the cumulus should be considered as described below (also see section "Ethical Concerns").

Should We Inseminate Fewer Than Six Oocytes, When There Is Choice?

In cases where a patient decided to freeze a portion of her oocytes, the number remaining for insemination will determine whether IVF or split-ICSI should be performed (see section "Oocyte Number Recovered from the Patient: If <6 Oocytes"). It should also be taken into account that oocyte cryopreservation requires decumulation of oocytes, which will necessitate ICSI for subsequent fertilization cycles.

Oocytes Maturity

Upon oocyte retrieval, embryologists mostly have to rely on the status of the cumulus to estimate the maturity status of the oocytes [39]. However, when a patient has fewer than 6 oocytes, the preferred protocol of many labs and as recommended by our criteria is to do all ICSI. When a significant percentage of those oocytes are immature (≥50%), this decision may prove complicated. Many laboratories by default denude all the oocytes in a small cohort, while standard practice for IVF requires an intact OCC, since inseminating denuded oocytes typically result in low fertilization rates (personal communication). However, Taylor et al. [43] found that IVF resulted in higher fertilization rates in sibling oocytes with increased immaturity. This is most likely because IVF gives the oocyte time to mature and be fertilized by the already-present sperm once it has matured, as conventional IVF insemination is typically performed overnight and provides a longer time window during which immature oocytes can mature, resulting in optimization of sperm-oocyte interaction and fertilization. With a smaller number of oocytes, each should be examined individually based on cumulus appearance and possibly for the presence of a polar body (see above). For those that are immature, IVF may yield better fertilization. Should these immature oocytes be denuded prior to determining maturation, they are typically cultured overnight to perform day 1 ICSI. However, this means that the oocyte may mature several hours before ICSI will be performed, possibly missing the window in which it can be successfully fertilized, emphasizing the importance of cumulus evaluation prior to denuding.

Should the oocytes be denuded and then it is discovered that a high percentage are immature, IVF can still be attempted, as some groups achieved good fertilization rates after inseminating denuded oocytes [44–46].

When Previous Insemination Was Only ICSI

This question is asked subsequent to whether it is the patient's first cycle. If it is not the first cycle and previously only ICSI was performed, there are no data available on IVF for those patients. There should thus not be a preference toward either technique without considering the factors downstream in Fig. 32.1. Essentially, neither ICSI nor IVF will be penalized on previous cycles. If the patient previously had both ICSI and IVF, the outcome of each technique in those cycles should be compared, as discussed in the next section.

How to Divide Oocytes for Insemination: IVF≥ICSI?

This question is to find out if the previous outcome of IVF was equal to or better than that of ICSI when considering fertilization, development, and the number and quality of resulting embryos. In patients that had cycles where IVF and ICSI were performed, embryologists have a considerable advantage in knowing the outcome of both techniques for the specific couple. Generally, the technique that previously proved more successful should be repeated, providing oocyte and sperm parameters are sufficient. This should be determined not only by fertilization rates but also by embryo quality, as the end goal is to obtain the largest number of good-quality embryos. If these outcomes were equal, the decision should lean more in favor of IVF, as it is a less invasive technique that is easier to perform and generally a more natural process (see above).

Other Factors

Apart from considerations outlined in previous sections, there are other factors that may influence the decision-making process that is external to the laboratory. These include issues that can be very patient specific and may essentially override the decision that the lab would typically make based on the flow chart (Fig. 32.1). Some programs may choose not to treat patients with criteria that negatively affect pregnancy rates. This is where science, ethics, and human emotions become merged to create very complicated situations for the clinics as well as the patients. These factors are discussed below:

Costs

As with most labor-intensive medical care, infertility treatment is not cheap. While most essential procedures are covered, at least partially, by medical insurance, this is not the case with most nonessential issues, such as infertility. Whether or not insurance will cover in vitro treatment is dependent on state and the specific insurance company and plan at hand. The cost schedule also varies greatly between different laboratories. Therefore, regardless of the diagnosis and recommended treatment, it should be confirmed that the patient has sufficient funds for the chosen procedure. Unfortunately, some patients will not be able to afford certain treatment plans. In general, ICSI is more expensive than IVF, whereas labs may charge for both procedures should split-ICSI be performed. Therefore, the choice of insemination method may sometimes be made purely on the financial

capabilities of the patient. This information should be discussed with the patient prior to start of treatment.

Ethical Concerns

IVF has always been, and will continue to be, a very controversial subject. This is especially true when religious beliefs are considered. Some patients are against the ICSI procedure since it is essentially in the hands of another human being to choose which sperm fertilizes the oocyte. These patients often request IVF only, regardless of oocyte numbers and sperm quality. The laboratory should always respect these requests, even if they may lower success rates. Other patients may not want to have too many embryos produced, since they do not feel comfortable with discarding or donating extra embryos and do not want the burden of having to deal with a large number of frozen embryos. In such cases, patients may want to freeze all oocytes except for a small number for insemination. Again, this will affect the choice of which procedure to use.

Technical Competence of Staff

Individual laboratories have staff with different skill levels. Each group should calculate their own fertilization and embryo development rates for ICSI and IVF. This is especially important for new, less-experienced groups. If there is a big difference in competence for the two procedures, the embryologists may want to adjust the ratio in favor of the one in which they are more proficient until they become more experienced and increase success rates with the other procedure. Well-trained, competent embryologists are key to obtaining desired results. It should also be considered that ICSI is labor intensive and therefore increases the workload on the lab. Incorporating IVF may relieve some of this stress in growing programs and give technicians extra time to improve other techniques. Naturally, decreasing workload should never be the sole motivation for choosing IVF, especially if it could lower the patient's chance of pregnancy.

Literature of IVF and ICSI Outcomes

Although often preliminary in nature, literature exists that indicates differences in the developmental potential of children conceived by ICSI and IVF [33–36]. There are also studies that suggest differences in the sex ratio obtained from the two procedures [47]. Furthermore, a wealth of often-inaccurate information is available on the Internet. Patients tend to research such topics and may approach the clinic with strong opinions about the different aspects of their treatment.

Influence of Friends and Family Members

Patients going through infertility treatment tend to share their experiences with family members and friends to get opinions from people they trust. Sometimes, these confidants may have gone through treatments themselves, or know people who have. In such cases, their opinions may carry a significant weight in the patient's decision of treatment. This can have an enormous implication of the preferred treatment choice. For example, a patient's sister may have had IVF and became pregnant on her first cycle. Thus, the patient wants the exact same treatment, although her oocyte and semen qualities are far inferior or superior to those of her younger sister. Despite the effort of the doctor and embryologist to better educate and advise the patient, she may not change her mind, which means that she will receive IVF or ICSI while she would probably have better chances of conceiving with ICSI (or it would not be necessary to perform ICSI).

Conclusions

Our objective with this chapter was to give a brief overview of the conventional IVF procedure and some of the factors that determine success. The decision-making process of whether to do conventional IVF, ICSI, or split-ICSI is, as discussed above, very complex. We presented the influencing factors in a manner that allows thorough evaluation of every case, resulting in an educated, well thought through decision of which insemination method to use. Subsequently (mostly to help us wrap our heads around the subject in preparation for writing this chapter), we developed a flow chart to indicate step by step how a decision is reached (Fig. 32.1). We believe it will assist embryologists in deciding which method of fertilization to use in their labs. The flow chart should be especially valuable for new, less-experienced groups that are considering both ICSI and IVF, but are unsure of how to implement both techniques into their treatment plans. We also hope that it will stimulate laboratories that are doing strictly ICSI to consider IVF and split-ICSI, as it may benefit both the patient and the lab by improving success rates, reducing costs, and minimizing workload.

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Intracytoplasmic Sperm Injection

33

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Abstract

Intracytoplasmic sperm injection (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 the sea urchin, in the mouse, and later in hamster eggs. This micromanipulation approach was also plagued by oocyte injury and lysis, with only about 30% of injected mouse eggs surviving the procedure. 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 or by exposure to A23187. The first ICSI offspring were obtained in the rabbit following the transfer of sperm-injected eggs into the oviduct of a pseudopregnant female, with a bovine live birth reported soon thereafter. Although applied to human gametes some years earlier, the first human pregnancies from ICSI was established only in 1992, and since then, hundreds of thousands of ICSI babies have been born. 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 standard IVF with apparently normal gametes.

Keywords

Intracytoplasmic sperm injection • Microsurgical epididymal sperm aspiration • Testicular sperm extraction • Microdissection testicular sperm extraction • Oocyte collection • Superovulation • ICSI procedure • ICSI technique

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Intracytoplasmic sperm injection (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 the sea urchin [1], in the mouse [2], and later in hamster eggs [3]. This micromanipulation approach was also plagued by oocyte injury and lysis [4], with only about 30% of injected mouse eggs surviving the procedure [5]. 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 [6] or by exposure to A23187 [7, 8].

The first ICSI offspring were obtained in the rabbit following the transfer of sperm-injected eggs into the oviduct

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of a pseudopregnant female [9], with a bovine live birth reported soon thereafter [10]. Although applied to human gametes some years earlier [11], the first human pregnancies from ICSI was established only in 1992 [12], and since then, hundreds of thousands of ICSI babies have been born [13–15].

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 standard in vitro fertilization (IVF) with apparently normal gametes [16, 17].

Indications for Intracytoplasmic Sperm Injection

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.

When initial sperm concentration in the ejaculate is $<5 \times 10^6$/mL, the likelihood of fertilization with standard IVF is significantly reduced [18], 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 because of a hardening of the zona pellucida [19] or when oocytes reveal ooplasmic inclusions [20, 21]. Abnormalities of the zona pellucida prevent sperm fusion with the oolemma [22], thus justifying sperm injection. In most instances, however, failure of fertilization is due to coexisting sperm abnormalities presenting ICSI as the only treatment option [13].

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 [3]. Although IVF of human oocytes was accomplished in men with epididymal spermatozoa recovered in cases of obstructive azoospermia [23, 24], only with the advent of ICSI was it possible to obtain consistent fertilization with them [16, 25, 26]. Testicular biopsy was employed to obtain sperm cells from men who had a scarred epididymis, and therefore, no chance of retrieval through that route [27–29]. However, the therapeutic possibilities of ICSI go even further since immotile testicular spermatozoa and even spermatids have been successfully used [30].

Some men produce only round-headed spermatozoa which have no acrosome and can neither bind to nor penetrate zona-free hamster oocytes [31, 32]. However, ICSI has enabled even such acrosomeless spermatozoa to establish pregnancies [33–37]. 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 [38]. 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 [39–41].

ICSI has also made possible a more consistent fertilization of cryopreserved oocytes [42]—overcoming the problem that freezing can lead to a premature exocytosis of cortical granules, resulting in zona hardening and inhibition of natural sperm penetration [43–46]. 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 enhances the number of fertilizable oocytes and so embryos available for screening [47].

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 1/1,000 acts of unprotected intercourse [48] and even fewer in HCV-infected patients [49]. 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 transmission of the virus. In such cases, intrauterine insemination (IUI) with spermatozoa processed by double gradient centrifugation followed by swim up has been the preferred method of treating serodiscordant couples with an HIV-1-infected male partner [50]. However, the use of ICSI has been proposed by several groups because of its negligible semen exposure, thereby reducing the risk of viral transmission [51, 52]. Advantages of ICSI over IUI also include the considerably higher success rate [52], requiring fewer attempts to achieve pregnancy while reducing viral exposure [53]. Fortunately, so far, no seroconversions have been reported following ART treatments including IUIs [54].

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) [55]. Such cases of spermatogenic arrest have necessarily involved the injection of immature spermatozoa or even spermatogonia [29, 30, 56, 57]. 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.

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, NY, USA)
- Vibration-free table (Newport Research Corporation, Irvine, CA, USA)
- Custom-designed horseshoe-shaped heated stage (Easteach Laboratory, Centereach, NY, USA)
- Microtools (Conception Technologies, San Diego, CA, USA)
  - Injection pipette (IC-SPN-30; 4–6 μ ID)
  - Injection pipette (IC-C1; 5–6 μ ID)
  - Holding pipette (HP-120-30)
- Oocyte transfer and denudation pipettes (hand-pulled, flame-polished Pasteur pipettes)
- ICSI dish (BD Falcon 351006; Becton, Dickinson and Co., Franklin Lakes, NJ, USA)
  - Stereomicroscope (SZX12, Olympus America Inc., Center Valley, PA, USA)
  - Distriman® Repetitive Pipette (F164001, Gilson, Inc., Middleton, WI, USA)—ICSI medium dispenser
  - AutoRep E™ Electronic Repeating Pipette (AR-E1, Rainin Instrument LLC, Oakland, CA, USA)—oil dispenser
  - Pipetman P20 (F123600, Gilson, Inc.)—polyvinylpyrrolidone (PVP) and sperm loading

Reagents

- ICSI Cumulase® (16125000, MediCult, Origio, Mt. Laurel, NJ, USA)
- Embryo culture medium (home-brew, modified Cornell medium based on G1™ and G2™ components [58, 59]).
- ICSI injection medium (G-MOPS™ [10129] supplemented with 6% G-MM™ [10038], Vitrolife, Inc., Englewood, CO, USA).
- Tissue culture oil (ART-4008, SAGE In Vitro Fertilization, Inc. A CooperSurgical Company, Trumbull, CT, USA)
- 7% PVP with HSA (90121, Irvine Scientific, Santa Ana, CA, USA)

Sperm Preparation

Ejaculated Sample

Semen samples are collected by masturbation after at least 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 [16].

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. [60]. Evaluations are carried out by spreading 10 μL of semen or sperm suspension on prestained slides (Cell-Vu®, Prestained 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 $<20\times10^6$/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 processed by centrifugation at $600\times g$ for 5 min in HTF medium supplemented with 0.4% HSA. Semen samples with $<5\times10^6$/mL spermatozoa or <20% motile spermatozoa are rinsed in a home-brew medium by a single centrifugation at $600\times g$ 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 $300\times g$ for 20 min when samples have a sperm density of $<5\times10^6$/mL spermatozoa and <20% motile spermatozoa. The sperm-rich fraction is rinsed by adding 4 mL of culture medium and centrifuged at 500–1,800 × *g* for 5 min to remove silica gel particles. For spermatozoa with poor kinetic characteristics, the sperm suspension is exposed to a 3 mM solution of pentoxifylline for 15 min and centrifuged one more time. The concentration of the assessed sperm suspension is adjusted to $1–1.5\times10^6$/mL, when necessary, by the addition of HTF medium and, subsequently, incubated at 37°C in 5% CO_2 in air.

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 maldevelopment 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 [61, 62]. The only method to retrieve spermatozoa from this form of azoospermia is a direct extraction of sperm or germ cells from a testis.

Microsurgical Epididymal Sperm Aspiration

The infertility of men due to irreparable obstructive azoospermia has been treated successfully by MESA [63, 64] or percutaneous testicular retrieval of spermatozoa [65]. 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 [16, 66].

Testicular Sperm Extraction

In the case of men with non-obstructive azoospermia, spermatozoa are retrieved directly from the testis in a procedure called testicular sperm extraction (TESE). 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 subtunical testicular blood supply with biopsy incisions made in avascular regions [63].

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 15-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 supernatant subsequently centrifuged at 500–1,800 × *g* for 5 min, an reevaluated for presence of spermatozoa. At times, it is possible to identify individual and enlarged seminiferous tubules that presumably are nesting 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.

Microdissection Testicular Sperm Extraction

In cases of non-obstructive azoospermia, the procedure for direct microscopic identification of functioning seminiferous tubules is referred to as microdissection testicular sperm extraction (micro-TESE) [67]. 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. 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 testes sutured.

When no spermatozoa are identified, testicular tissue are placed in 1 mL of prewarmed medium supplemented with 5% HSA, 1.6 mM $CaCl_2$ (Sigma Chemical Co., St Louis, MO), 25 μg/mL DNase (Sigma Chemical Co.), and 1,000 IU/mL collagenase type IV (Sigma Chemical Co.) [68]. 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 [69, 70]. 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 resuspended in 100–500 μL of sperm cell medium.

Oocyte Collection

Superovulation is performed by administration of gonadotropins in association with agonist or antagonist protocol [71]. 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.

Intracytoplasmic Sperm Injection Procedure

Loading the Microtools

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, 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 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.

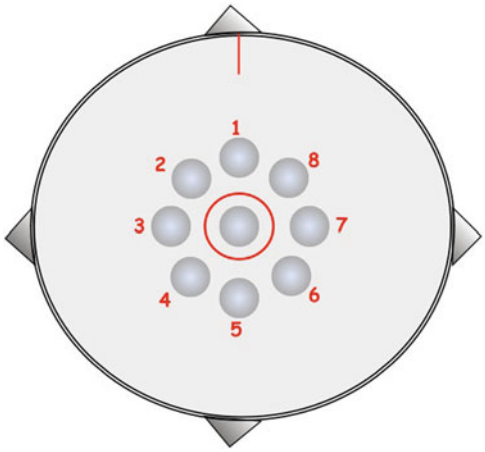

Fig. 33.1 The drawing depicts a Petri dish containing the droplets of medium (8 μL) and sequentially numbered going counter clockwise. The center droplet is removed and replaced with PVP and sperm while in each surrounding droplets, an individual oocyte will be placed

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. 33.1). 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 drawn around the central drop, and the drops are sequentially numbered starting from the 12 o'clock position, moving counter clockwise. This allows easy navigation between droplets during ICSI. ICSI dishes are stored at 37°C until use.

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.

Sperm Immobilization

Position the spermatozoon at 90° with respect to the pipettes' tip, 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. 33.2a–c). If initially unsuccessful, the procedure is repeated until the tail is clearly kinked, looped, or convoluted (Fig. 33.2d). 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 z-axis

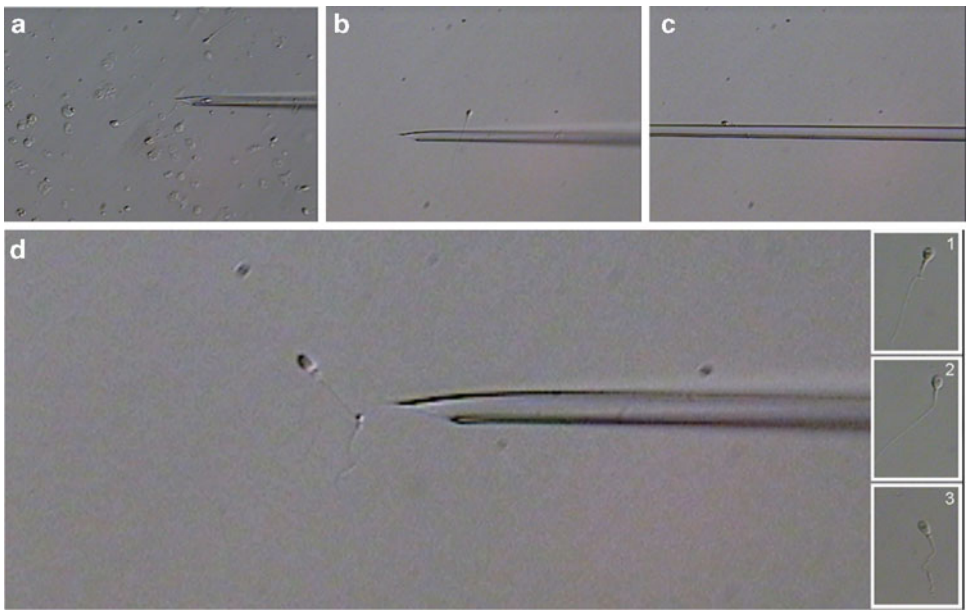

Fig. 33.2 Aggressive immobilization of a testicular spermatozoon prior to intracytoplasmic sperm injection (ICSI). (**a**) A single spermatozoon displaying a satisfactory morphology is aspirated from a sperm droplet. Such droplets are made without PVP in cases where sperm count and motility are poor. (**b**) The chosen spermatozoon is then moved into the PVP droplet, where the injection pipette is gently lowered onto the sperm tail principal piece, compressing it. Even though the spermatozoon does not display motility, immobilization is still carried out to ensure the best chance of fertilization, since an active damage to the 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)

knob 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 vs. culture medium) may allow the sperm to move distally into the pipette and become loose.

Injection

To find the egg, the magnification is briefly lowered to ×200, and once the egg is centrally in the field, the magnification is 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 rarefaction (or polar granularity) (Fig. 33.3a). 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. 33.3b–d). At this point, a break in the membrane should occur at the approximate center of the egg (Fig. 33.3e). 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. 33.3f), as well as by the proximal flow of cytoplasmic organelles and the spermatozoon back into the pipette. The spermatozoon is then ejected with the cytoplasmic component (Fig. 33.3g). 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. 33.3h). 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 withdrawing 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. 33.3i). 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

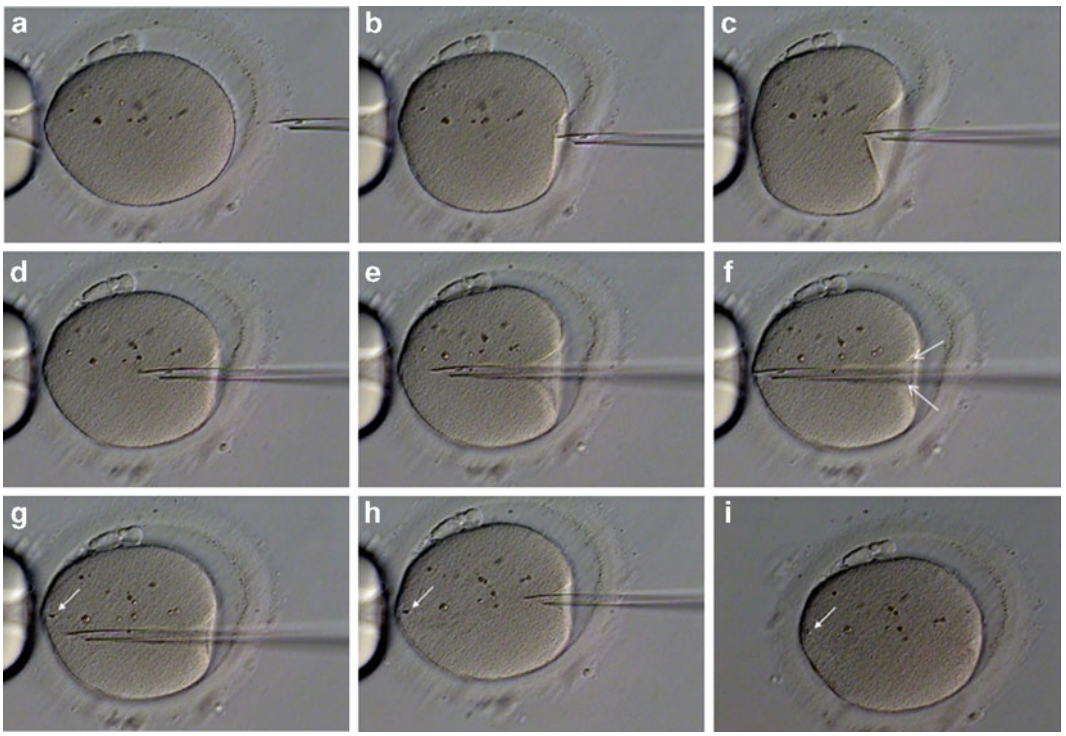

Fig. 33.3 ICSI of a human oocyte. (**a**) The oolemma at the 3 o'clock portion of the oocyte is brought into focus, as are the holding (*left*) and injection pipette (*right*) tips. Suction is applied on the holding pipette to stabilize the oocyte, and the spermatozoon is advanced to the tip of the injection 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 (*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

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 seconds.

Embryo Development and Clinical Outcome

In the last 16 years at Cornell, we have performed a total of 29,191 ART cycles with an average maternal age for IVF of 37.6 ± 4 years and for ICSI of 35.8 ± 5 years and mean paternal ages of 39.6 ± 6 and 40.8 ± 8 years, respectively. Of those cycles, 36.0% (10,503) included the standard in vitro insemination of 106,931 oocytes from 5,943 couples with a fertilization rate of 58.9% and a clinical pregnancy rate of 40.4%, 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 10,922 couples treated by ICSI, the average number of oocytes retrieved was 10.8 per cycle ($n = 201{,}709$), of which 156,377 (77.5%) were at MII and, therefore, subjected to ICSI. Of the oocytes injected, 94.7% (148,089/156,377) survived and 114,288 (73.1%) developed two pronuclei (PN). Of the oocytes that were abnormally fertilized, 4,066 (2.6%) displayed 1PN and 5,004 (3.2%) 3PN.

The types of mature spermatozoa included 15,199 ejaculated samples, 1,531 cryopreserved, 79 obtained by electroejaculation, and 29 by bladder catheterization (Table 33.1). Among the collection/cryopreservation methods, fertilization rates ranged from 73.5 to 82.5%. 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 46.0% was satisfactory but lower than that with ejaculated spermatozoa ($P = 0.0001$) (Table 33.2). 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

Table 33.1 ICSI outcome according to semen source

Semen origin	Cycles	Fertilization (%)	Clinical pregnancies (%)
Fresh ejaculate	15,199	92,876/124,774 (74.4)	5,712 (37.6)
Frozen ejaculate	1,531	9,296/12,643 (73.5)	547 (35.7)
Electroejaculate	70	517/681 (75.9)	34 (48.6)
Frozen electroejaculate	9	78/102 (76.5)	3 (33.3)
Retrograde ejaculate	29	231/280 (82.5)	9 (31.0)

Table 33.2 Fertilization and pregnancy rates according to the origin of spermatozoa

	Spermatozoa	
No. of	Ejaculated	Surgically retrieved
Cycles	16,838	1,846
Maternal age (M years ± SD)	37.3 ± 5[a]	34.2 ± 5[a]
Fertilization (%)	102,998/138,480 (74.4)[b]	11,290/17,897 (63.1)[b]
Clinical pregnancies (%)	6,305 (37.5)[c]	849 (46.0)[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 33.3 Pregnancy characteristics of 18,684 ICSI cycles

	No. of (%)	Negative outcomes	
ICSI cycles	18,684		
Embryo replacements	17,337		
Positive ßhCGs	9,435 (50.5)		
		Biochemical pregnancies	1,521
		Blighted ova	681
		Ectopic pregnancies	79
Patients with fetal heartbeats	7,154 (38.3)		
		Miscarriages/ therapeutic abortions	708
Deliveries and ongoing pregnancies	6,446		

acquired, had no effect on fertilization after ICSI giving an overall rate of 70.5% and a clinical pregnancy rate of 53.5%. However, after cryopreservation of epididymal spermatozoa, the clinical pregnancy was dramatically reduced from 62.3 to 48.2% ($P=0.0001$).

In testicular extraction cases, with the compromised spermatogenesis typical of the non-obstructed azoospermic patient, the spermatozoa recovered generated a fertilization rate of 56.9% and a clinical pregnancy rate of 41.2%. When comparing fresh vs. cryopreserved spermatozoa in testicular cycles, the clinical pregnancy rate was comparable between the two groups (42.6 vs. 35.6%).

Of the 18,684 ICSI cycles analyzed, 9,435 resulted in a positive ßhCG (50.5%), and in about 38% of all cycles, the presence of at least one fetal heartbeat was observed (Table 33.3). Among the positive ßhCG, 1,521 (16.1%) were biochemical pregnancies, 681 (7.2%) were anembryonic, and 79 (0.8%) were ectopic. Among the 7,154 cycles with a fetal heart, 708 spontaneously miscarried. This left an ongoing pregnancy rate of 34.5% per retrieval (6,446/18,684) and 37.5% per embryo replacement procedure (6,446/17,337).

When 17,700 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 33.4) 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 5,922 ICSI patients delivered 8,105 babies comprising 4,051 males and 3,994 females (with 60 unknown genders). A total of 3.3% (268) exhibited congenital abnormalities at birth, of which 130 were major and 138 were minor. IVF children ($n=4,999$) had a 3.1% overall malformation rate (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; 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.

Table 33.4 The relationship of maternal age to ICSI outcome

	Maternal age		
No. of cycles with (%)	<35	35–39	≥40
Cycles	5,755	6,412	5,533
(a) Embryo replacement	5,318	5,956	5,091
(b) Clinical pregnancy (+FHB) (% on a)	2,823 (53.1)[d]	2,535 (42.6)[d]	1,323 (26.0)[d]
(c) Delivery and ongoing pregnancy (% on a)	2,670 (50.2)[e]	2,297 (38.4)[e]	795,902 (18.8)[e]

[d,e] χ^2, 3×2, 2 *df*, effect of maternal age on pregnancy outcome, $P=0.0001$

Intracytoplasmic Sperm Injection 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 testes. 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 [33, 72]. The reasons for these few failures remain unclear, but probably involve either poor sperm viability or an asynchronous oocyte maturation [47, 73].

Some men carrying acrosomeless (also known as globozoospermic) spermatozoa cannot achieve syngamy even after ICSI [33, 74, 75]. However, successful fertilization, pregnancy, and offspring have resulted after treating the oocytes post-ICSI by either a chemical agent [34, 75–77] or an electrical pulse [76, 78]. Similarly, the chances of fertilization in these problematic cases may sometimes be enhanced by utilizing sperm-derived activating extracts or calcium-releasing compounds [79, 80]. Oocyte activation agents include but not limited to calcium ionophore [7], electrostimulation [76], and strontium [77]. 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 [34], 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, 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 [81, 82].

The inability to activate an egg has been attributed to the absence of a specific sperm protein that has been investigated in many occasions [79, 83, 84]. 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 [85, 86]. As an interesting correlate, the PLCζ1 isoform was undetectable in sperm of men that experienced fertilization failure with ICSI [87]. 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

Finally, it must be emphasized 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 [34, 36, 88, 89].

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 [79, 90, 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 [92]. 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 [93].

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 [94]. 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 [95] or cognitive development [96]. Therefore, follow-up of ART children is highly recommended and is being increasingly applied [97–100]. Parent-administered questionnaires have been proposed as way of doing this, since a routine office assessment is costly and time-consuming [15, 101, 102].

The specific concerns in regard to ICSI, whether real or theoretical [103–106], 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 [107], a recurrent reduction in birth weight [108], certain rare syndromes related to imprinting errors [109–114], and even a higher frequency of some cancers [115]. 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 [106]. A systematic survey aimed at clarifying imprinting issues suggests that only Beckwith–Wiedemann syndrome (BWS) may have such a link [116].

A shorter gestational age and a lower birth weight have been noted in ART pregnancies irrespective of the conception method [95, 108]. The basis for this could lie in an unclear medical history [117], in the fact that a singleton may result from early in utero loss of a twin [118] or where an ART delivery is performed at the slightest sign of fetal distress [119]. 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 [15]. Similarly, in the study of Lin et al. [120] 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 [107]. However, this study failed to correct for maternal age and for other patient characteristics [121], for the presence of male factor [122], and even in its classification methods [123]. 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 [124]. In another series, ICSI vs. naturally conceived singletons that were matched for maternal age displayed no difference in neonatal outcomes [120], and this was clearly the case when the outcomes of neonates generated by different artificial conception procedure, ICSI vs. IVF, were investigated [125].

In this connection, Bowen et al. [96] 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 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 [126] 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 ($P<0.05$) [14]. This study revealed that ICSI children were indeed characterized by a lower birth weight ($P<0.05$) and 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 [127]. Another study comprising 1,500 children from five 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 [98]. The same Brussels group confirmed a malformation level of 10.0 vs. 3.4% in the same cohort of NC children once they reached 8 years of age [128]. These three additional malformations included of 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 [129]. 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.

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 [115]. A Swedish registry-based study identified five cases of Langerhan's histiocytosis among 16,280 IVF infants in comparison to the 0.9 expected cases in the general population [130]. 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 also been posed about the effect of the embryo culture medium on the health of ART children in general and whether this can induce imprinting disorders [131]. Imprinting disorders such as Angelman syndrome (AS) [109, 113] and BWS [109, 132, 133] have been described in connection to assisted reproductive technologies. A survey of 1,000 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 [116]. Both syndromes have been found to be largely due to epigenetic defects rather than a genetic mutation or uniparental disomy [134], thus suggesting that some aspect of the ARTs may be responsible.

In a recent study in mice, Wilson et al. [135] 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 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 [136]. 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 postimplantation 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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Mechanism of Human Oocyte Activation During ICSI and Methodology for Overcoming Low or Failed Fertilization

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Abstract

The mechanism of fertilization after ICSI is markedly different from that during natural fertilization. During normal fertilization process, sperm–oocyte fusion is followed by incorporation in the cytoplasm of a demembranated, "naked" sperm nucleus, which immediately becomes accessible for ooplasmic factors, including thiol-reducing agents. Following ICSI, not a "naked" sperm nucleus but the whole sperm cell, enclosed in its membrane, is exposed to the ooplasm.

Keywords

Human oocyte activation • ICSI oocyte activation • Failed fertilization • Mechanism of human oocyte activation • ICSI protocols

Mechanism of Human Oocyte Activation During Fertilization Using Intracytoplasmic Sperm Injection

The mechanism of fertilization after intracytoplasmic sperm injection (ICSI) is markedly different from that during natural fertilization. Whereas during normal fertilization process, sperm–oocyte fusion is followed by incorporation in the cytoplasm of a demembranated, "naked" sperm nucleus, which immediately becomes accessible for ooplasmic factors, including thiol-reducing agents [1]. Following ICSI, not a "naked" sperm nucleus but the whole sperm cell, enclosed in its membrane, is exposed to the ooplasm [2, 3].

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Sperm Plasma Membrane Damage

It has been demonstrated that whole sperm cells behave in a different way than partially demembranated sperm cells in cytoplasmic cell extracts [4]. If an intact spermatozoon injected into the ooplasm, there will be no interaction between the spermatozoon and the oocyte. This is because ooplasm does not have an enzyme to digest sperm plasma membrane to enable interaction (Dozortsev, unpublished). Therefore, sperm plasma membrane has to be damaged prior to the fertilization. Even though sperm cells appear to have distinctive compartments, such as a head, midpiece, and tail, they have only one plasma membrane covering the entire cell. Therefore, sperm plasma membrane can be damaged in any area, in order to enable sperm–egg interaction after injection. Usually, during ICSI, the sperm plasma membrane is damaged by "touching" tail [5], which in reality is pressing the sperm tail with a glass pipette, until the sperm membrane damage becomes sufficient for the membrane to loose its ability to maintain cell integrity, which leads to disabling Na/K pump and loosing membrane potential. To the observer, this event is marked by the sperm ceasing movement. The sperm membrane damage can also be revealed using vital

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staining technique, such as Eosin B [5] as can be seen on the figure further in the text. Vital staining is based on the intact membrane ability to exclude Eosin B. But once the membrane loses its charge, Eosin B migrates inside of the cell and stains the nucleus.

Effect of PVP

PVP, which is usually present in the culture medium, has the ability to act as a membrane's "band-aid" and prevents the stain migration into the nucleus. The plasma membrane damage induced by "touching" tail does not allow anything from the sperm cell to "leak" into surrounding medium. Indeed, the sperm cells retain full capacity to fertilize an oocyte for several hours following immobilization [6].

Initial Sperm Nucleus Swelling

Within minutes after injection, the sperm nucleus becomes accessible for the thiol-reducing agents (primarily glutathione—GSH) of the ooplasm and swells [7]. It is important to understand that this initial swelling is not under the same control as nuclear decondensation necessary for pronuclei formation. This swelling can also be easily duplicated in vitro using thiol-reducing agent—dithiothreitol (DTT).

The most abundant low-molecular-weight nonprotein thiol exists in two forms: reduced GSH and GSH disulfide (GSSG). It has been clearly shown that GSH is a disulfide bond reducer present in the cytoplasm of oocytes [1, 8]. Oocytes can synthesize GSH (tripeptide-glutamyl-cysteinyl-glycine, GSH) during the first meiosis. Oocyte-derived GSH seems to assure the reduction of disulfide bonds in sperm nucleus. This, in turn, promotes nuclear decondensation for male pronucleus formation during fertilization (reviewed by Sutovsky and Schatten [9]). Thus, GSH provides the reducing power to initiate chromatin decondensation, prior to the male pronucleus formation [10, 11]. Depletion of endogenous GSH by a specific inhibitor of GSH synthesis during bovine oocyte maturation blocks the formation of a male pronucleus and prevents the assembly of sperm aster microtubules [9]. The elevated levels of oocyte GSH can enhance male pronuclear formation after IVF [11]. If an excessive amount of PVP is injected with the sperm cell, for the reasons described above, sperm and egg interaction can be delayed.

Sperm-Associated Oocyte-Activating-Factor Release

The initial swelling of the sperm nucleus ruptures sperm plasma membrane and enables the release of the sperm-associated, oocyte-activating factor—PLC zeta—into the ooplasm. The entire mechanism of fertilization after ICSI can be summarized as the following:

1. Sperm plasma membrane damage is induced by "touching" tail, but the damage is masked as long as PVP is present.
2. Following injection, PVP is diluted and reduced GSH and GSH disulfide (GSSG) enter the sperm nucleus inducing sperm nucleus swelling within a few minutes.
3. The nuclear swelling ruptures sperm plasma membrane, enabling the contact between nucleus-bound PLC zeta and ooplasm, inducing oocyte activation and leading to chromatin decondensation. Most likely, due to the variations in the sperm plasma membrane damage and the amount of PVP injected, there is a large disparity in beginning Ca^{2+} oscillations among oocytes in the same cohort. Unlike during natural fertilization, when oscillations begin almost immediately after fertilization, oscillations after ICSI may begin within minutes or hours after injection.

Experimental and Theoretical Basis for Artificial Activation of Human Oocytes

Mechanisms of Activation Failure

Cases in which all MII oocytes become fertilized following ICSI are not common. In fact, the average fertilization rate after ICSI is usually below 80%. Cytological analysis of oocytes that failed to fertilize usually reveals that spermatozoon was interacting with the ooplasm but failed to trigger the activation [7]. Ovulated human oocytes are extremely resilient to parthenogenetic activation by sham-ICSI [12]. Therefore, even if fertilization rate is very low, it is usually due to the presence of some activating factor in the spermatozoon. Thus, the true deficiency of the activating factor should only be suspected when less than 10% of the oocytes failed to fertilize.

In most cases, failure of fertilization can be forecasted based on sperm morphology. It is very uncommon that a morphologically normal motile (live) spermatozoon fails to activate an oocyte due to activating factor deficiency. If activation fails in such case, the activation problem is most likely due to the oocyte.

At the same time, it is important to understand that morphological assessment of the sperm cells is not predictive of its activating potential. For example, acrosomeless (globospermic) spermatozoa in some cases will successfully activate human oocytes [13, 14], while in others, their injection will result in 100% failure of activation [15]. This may, to certain extent, correlate with acrosin-positive or acrosin-negative status of acrosomeless spermatozoa, which in its own turn seem to correlate with respectively the presence or absence of PLC zeta. Similarly, in the case of Kartagener's syndrome, immotile spermatozoa may still activate an oocyte [16].

Since there are strong evidences that PLC zeta is the sperm-derived activating factor, one could argue that using respective antibodies would provide a discriminating testing. However, due to morphological peculiarities of the sperm cell, in situ antibodies testing may often be nonconclusive. Therefore, functional testing, by injecting spermatozoa in question into the oocytes, seems to be the most feasible assay at this time [15].

Tests for Sperm-Activating Ability

Because sperm-activating factor (PLC zeta) is not species-specific [17–19], mouse, bovine as well as oocytes of other mammal oocytes can be used to test activating ability of human spermatozoa. Hamster eggs usually survive injection better, and they can be purchased frozen. However, they are expensive and may be activated by pricking itself, masking true activation capacity of a spermatozoon (although we were not able to activate by sham-ICSI any of the frozen-thawed hamster's eggs). Also, importantly, there is no clear benchmark activation rate has been established for activating potential of the human spermatozoa using hamster' eggs.

Mouse eggs, on the other hand, do not survive injection as well as hamster eggs, and we were not able to locate the source of frozen mouse eggs. Similarly to hamster, mouse eggs may also be activated by pricking, particularly as they age, and their sensitivity to parthenogenetic stimuli increases. Therefore, it is recommended that for the purpose of sperm activation testing, mice would be sacrificed shortly after expected ovulation time and used within 4 h after expected ovulation.

One clear advantage of mouse eggs is that their activation rate (excluding eggs which are damaged or were not injected correctly) following injection of normal human spermatozoa is 100%. Therefore, any deviation from this benchmark is strongly suggestive of activation factor deficiency. Even though the functional tests are available, their practical significance is not very high because usually the initial ICSI attempt will serve as a test and, as experience demonstrates, the underlying reason of failed fertilization will not affect the subsequent treatment modality.

Artificial Activating Factors

Development can be set in motion by a number of artificial stimuli, although their range is smaller than in mice. For example, ethyl alcohol, a very potent activator of mouse oocytes, is not able to induce activation of human oocytes, even 1-day old (Dozortsev et al., unpublished).

The majority of physical and chemical activating stimuli used for artificial activation in the humans elicit Ca^{2+} release into the cytoplasm, mimicking to different extent Ca^{2+} release taking place during natural fertilization [20]. The end point of activation is a physical destruction of cyclin B, which leads to inactivation of p34 kinase and drop in MPF activity [21]. Even though animal research, and in particular Ozil's work [22], assign the importance to the pattern of the Ca^{2+} oscillations during artificial activation, the relevance of activation pattern for human oocytes injected with the sperm is not certain. This is because in animals, the impact of artificial activation stimuli has been tested largely on parthenogenetic embryos, which do not generally develop well due to imprinting problems. In fact, in mammals, even when an oocyte is activated by sperm, but the male genome does not participate in development (gynogenesis), embryo development is usually poor and rarely survives far past implantation. Cloning experiments, where only a tiny minority of embryos develop to term [23], also illustrate an overwhelming importance of genetic makeup of the embryo over the activation modality.

One of the potent artificial activating agents of human oocytes—puromycin—has to be mentioned separately. This is because it does not cause Ca^{2+} release (Dozortsev, unpublished) and probably acts by suppressing synthesis of the cyclin B and by inhibiting its phosphorylation, rather than by its physical destruction. The point of no return in puromycin-activated oocytes is most likely the initiation of DNA synthesis (Dozortsev, unpublished). This observation has practical importance because it predicts that puromycin will act synergistically with Ca^{2+} engaging activation stimuli.

Indications for Artificial Oocyte Activation

Based on the current experience, fertilization failure of any etiology is an indication for artificial activation. Also, it is important to note that even though oocytes that failed to become fertilized following ICSI can be successfully activated on day 1 and many display two pronuclei and a second polar body, no pregnancy has been reported. Therefore, only fresh cases are the candidates for the procedure. Rescue of failed ICSI is likely to be futile not solely because of oocyte aging, since rescue ICSI following conventional insemination has been reported to result in pregnancy. The confounding factor for rescue activation is probably sperm chromatin deterioration to the extent that it prevents embryonic development.

Protocols Resulted in Live Birth After Artificial Activation

Considerations

As a general rule, activation stimulus has to be applied to the oocytes at the same time or shortly after sperm injection. If activation stimulus is applied before sperm injection, changes in the cytoskeleton and plasma membrane may make oocytes

more fragile and prone to damage. On the other hand, in the absence of an activation stimulus, injected sperm chromosomes quickly undergo premature chromosomes condensation, and the significant delay between injection and activation may lead to the loss of sperm chromosomes (accessory micronuclei, along side of the pronuclei may be seen in such case).

Another practical consideration is the timing of ICSI and activation relative to hCG administration. Oocytes generally become more susceptible to parthenogenetic activation as they age. However, it has been shown that optimal fertilization window is between 39 and 42 h after hCG [24]. Therefore, it would seem that the best time for artificial activation would be around 42 h after hCG. It should also be noted that if oocyte deficiency is suspected as an underlying reason of failed fertilization, delaying ICSI and activation until 45–47 h post-hCG may be desirable.

Specific Protocols

Reference: Rybouchkin et al. [25]
Diagnosis: Globozoospermia
Outcome: Ongoing pregnancy
Protocol: Four different procedures for assisted oocyte activation were applied to the donated oocytes:

1. 111 Vigorous aspiration of oocyte cytoplasm during sperm injection [6].
2. 121 Same as above protocol plus ionophore A23187 treatment (5 mM during 7 min) at 30 min after sperm injection.
3. 131 Injection of approximately 5pl of 0.1 M $CaCl_2$ along with a spermatozoon, followed by ionophore treatment at 30 min after ICSI (This procedure has been proposed by this study).
4. 141 The same as above plus with ionophore treatment 30 and 60 min after injection.

Reference: Kim et al. [26]
Diagnosis: Round-headed spermatozoa
Outcome: Fertilization rate, implantation, pregnancy, and delivery [21 of 35, 60%; two pronuclei in 18 of 21; three pronuclei in 3 of 21]
Protocol: Oocyte aspiration was performed with transvaginal ultrasound guidance. After 5 h, all oocytes were denuded enzymatically with 0.1% hyaluronidase (Sigma, St. Louis, MO) for 30–60 s, followed by mechanical denudation. Motile round-headed spermatozoa were injected into oocytes in metaphase II, and assisted oocyte activation was performed with calcium ionophore A23187 (Sigma). At approximately 16–18 h after injection, the presence of 2PN was recorded as a sign of fertilization.

Reference: Eldar-Geva et al. [27]
Diagnosis: Normozoospermic patient with previous repeated failed fertilization after ICSI (case report)
Outcome: Fertilization rates in three cycles were 4/6, 5/16, and 7/20 oocytes. Two pregnancies were achieved; the first ended with second trimester miscarriage due to fetal anomaly and the second with a delivery of three healthy babies.
Protocol: Within 1 h of injection, six oocytes were exposed to 10 mmol/L of ionophore A23187 in IVF-50 medium for 7 min at 37°C in 5% CO_2. The oocytes were then washed free of the ionophore through ten drops of fresh culture medium and incubated further as usual.

Reference: Chi et al. [28]
Diagnosis: Normozoospermic patient (case report)
Outcome: The fertilization rate of oocytes activated (12 of 15, 80.0%) was higher than that of the nonactivated oocytes (4 of 16, 25.0%). Twin pregnancy
Protocol: Thirty minutes after ICSI, the oocytes were exposed to 8 mmol/L calcium ionophore for 8 min and subsequently washed thoroughly in P1 medium

Reference: Dirican et al. [29]
Diagnosis: Two siblings with familial globozoospermia
Outcome: Fertilization rate in case 1 and 2 were 33.3 and 9.1%, respectively. Clinical pregnancies with healthy live births were observed.
Protocol: Oocytes were mechanically activated before ICSI.

Reference: Heindryckx et al. [30]
Diagnosis: Failed or low fertilization in previous ICSI cycles or who had well-known sperm-borne activation deficiencies such as globozoospermia
Outcome: High fertilization and acceptable pregnancy rates
Protocol: Oocytes were kept at 37°C in a 6% CO_2 air atmosphere in Cook Cleavage medium (Cook Ireland Ltd, Limerick, Ireland). For ICSI with AOA, spermatozoa resuspended in HEPES-buffered oocyte wash (Cook Ireland Ltd) and an equal volume of 8% polyvinylpyrrolidone (PVP ICSI-100, VitroLife Sweden AB, Kungsbacka, Sweden) was immobilized by pressing the tail to the bottom of the dish and was drawn up into an injection pipette, and the sperm head was kept at the very tip of the pipette. Then the pipette was moved to a drop of 0.1 mol/L $CaCl_2$, and an amount of $CaCl_2$ was aspirated into the injection pipette, which corresponded to the diameter of the oocyte. Oocytes were conventionally injected with the spermatozoa and $CaCl_2$ and kept in Cook Cleavage medium for 30 min. Injected oocytes were exposed for 10 min in the incubator to 10 mmol/L Ca^{2+} ionophore (Ionomycin, cat. no. 159611; MP Biomedicals) dissolved in Cook Cleavage medium and subsequently washed intensively and put in Cook Cleavage for 30 min in the incubator. Finally, ionophore treatment was repeated during 10 min, and after

intensive washing, oocytes were placed in Cook Cleavage medium for culture.

Reference: Nasr-Esfahani et al. [31]
Diagnosis: Severe teratozoospermia
Outcome: Improvement of fertilization and cleavage rates after AOA
Protocol: Oocytes were randomly divided into two groups: control and AOA. The injected oocytes in the control group were cultured in G1. The remaining oocytes were chemically activated by exposure to 10 mM ionomycin for 10 min.

Reference: Kyono et al. [32]
Diagnosis: Oocytes from patients with repeated fertilization failure
Outcome: Live birth
Protocol
Materials
- $SrCl_2 \cdot 6H_2O$: Sigma-Aldrich #255521
- Dolbecco's modified Eagle's medium (DMEM): GIBCO #21068

Method
1. $SrCl_2 \cdot 6H_2O$ stock solution
   We prepared 100 mM $SrCl_2$ stock solution in DMEM and stocked at −30°C.
2. The microdroplet of 10 mM $SrCl_2$ solution (in DMEM) was overlaid mineral oil and stored in 6% CO_2, 5% O_2, and 89.9% N_2 air condition overnight.

These oocytes were cultured in Universal IVF medium for 30 min and then activated in $SrCl_2$ (10 mmol/L), 10% synthetic serum supplement, and Dulbecco's modified Eagle's medium (Gibco, USA) 20 mL/drop under 6% CO_2, 5% O_2, and 89% N_2 under humidified conditions for 60 min.

Reference: Ahmady et al. [33]
Diagnosis: Nonviable testicular sperm is used for intracytoplasmic injection (ICSI).
Outcome: Full-term delivery
Protocol: The injected oocyte was incubated in G-Fert (Vitrolife, Englewood, Colo) medium containing 10 mg/mL calcium ionophore A23187 (stock solution 10 mg/mL in dimethyl sulfoxide A23187 (stock solution 10 mg/mL in dimethyl sulfoxide stored at 220°C; Sigma Chemical Co, St Louis, MO) for 10 min.

Reference: Heindryckx et al. [15]
Diagnosis: Patients with previously failed fertilization and globozoospermia
Outcome: After AOA, fertilization rates were 77 and 71% in the sperm- and oocyte-related groups, respectively. Five pregnancies were achieved in the globozoospermia group and three in cases of oocyte-related activation failure.
Protocol: Activation capacity was assessed by 2-cell formation (mouse oocyte activation test, MOAT). When no activation occurred, AOA was done by ICSI with $CaCl_2$ followed by a $CaCl_2$ (0.1 mol/L) ionophore (10 mmol/L ionophore) exposure.

Reference: Juan Chen et al. [34]
Diagnosis: Patients with fertilization failure or low fertilization rates
Outcome: Improve fertilization rates (78.8%) and embryo quality (41.5%) (17/41) in cases with fertilization failure after ICSI
Protocol: Oocytes were activated in calcium-free HTF medium containing 10% (v/v) SSS and 10 mM $SrCl_2$ (Sigma Chemical) for 60 min at 37°C and 5% CO_2 [35].

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Part VII

Micromanipulators and Micromanipulation

Hydraulic Manipulators for ICSI

35

Hubert Joris

Abstract

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.

Keywords

Hydraulic and electrical manipulators • Microtools • Micromanipulation systems • Intracytoplasmic sperm injection • IVF instruments

Micromanipulation of cells started more than a century ago. Already in 1859, a description of some kind of microdissector has 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 this 100 years between the first description of micromanipulation and the use of this technique for the study of fertilization events, the technologic 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 60s. 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. To transfer the movement from a macroscale to a microscale can be done in different ways. This can be done mechanically, hydraulically, and electronically. The use of mechanical and electronic manipulators and their use in ART is subject of different chapters.

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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.

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 its 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, 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.

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 tools can easily return to their starting position, e.g., 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)

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 exists 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

Fig. 35.2 Example of control unit of hydraulic manipulator with hanging joystick (courtesy of Nikon, Melville, NY, USA)

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.

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 has been changed. Hanging joysticks are now available for different

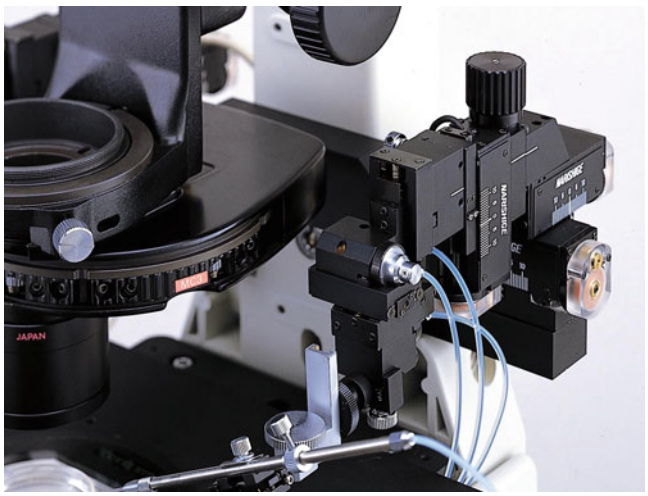

Fig. 35.3 Example of drive unit of hydraulic manipulator (connected to control unit by tubing) mounted on electrical coarse control manipulator and universal joint for mounting of the tool holder (courtesy of Nikon, Melville, NY, USA)

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 course 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 starting 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.

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 this 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 a procedure that has to be interrupted 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 for certain characteristics requiring 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 (start 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.

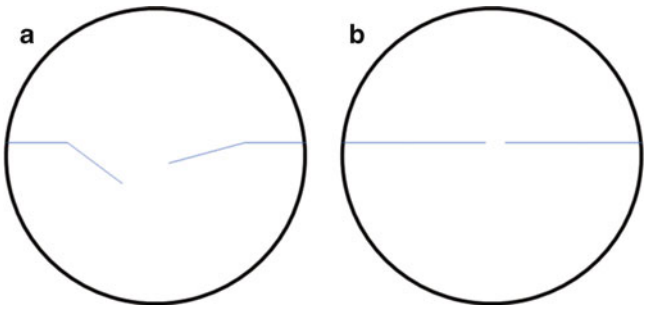

Fig. 35.4 Top view of microtools before initial alignment (**a**) and after initial alignment (**b**). Holding pipette on the left hand side, injection pipette on the right hand side

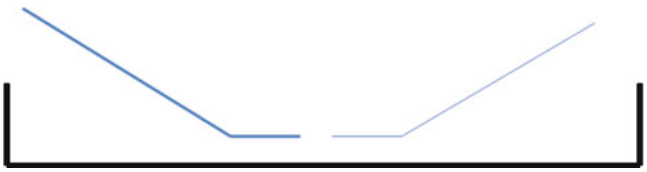

Fig. 35.5 Correct position of microtools at the start of the ICSI procedure. The bent tip is positioned horizontally

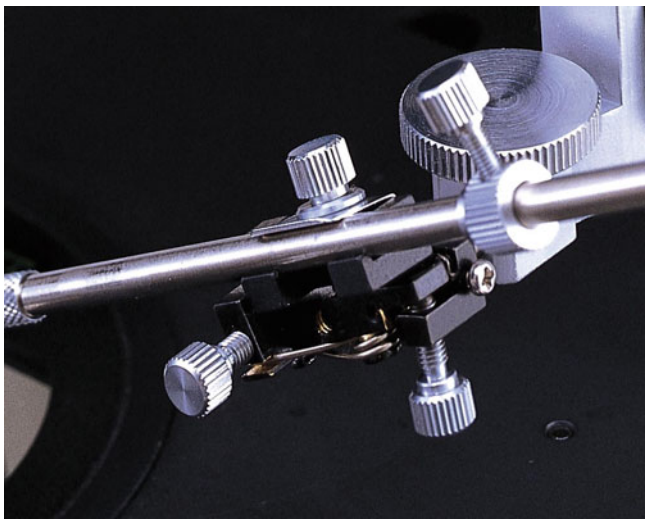

Fig. 35.6 Example of a more recent type of universal joint where changes in position can be made by simple rotation of screws with mounted tool holder (courtesy of Nikon, Melville, NY, USA)

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 of 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 starts. 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) is not always 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 once working at larger magnification.

The way the drive unit and universal joint are constructed and mounted allows positioning of the tool holder in almost unlimited different 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 of 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, number 2. This results in difficulties fixing the oocyte when it is the case for the holding

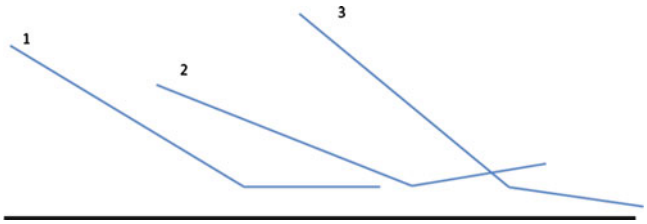

Fig. 35.7 Different positions of microtools. *1*: correct position; *2*: tip of the pipette cannot reach bottom of the dish; *3*: only the very end of the tip touches the bottom of the dish

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, number 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 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 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 details 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 authors' 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.

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Research Instruments Micromanipulators

36

Steven Fleming and Catherine Pretty

Abstract

Research Instruments micromanipulation systems incorporate purely mechanical micromanipulators combined with air-assisted microinjectors. This design concept increases their reliability, making them virtually maintenance-free. These apparently simple principles of design are deceptive, however, as they are supported by innovative features such as a laser-guided micropipette set-up device, one-touch pipette angle adjustment and touch-screen independent control of three built-in heated stages.

Keywords

IntegraTi • Research Instruments • Partial zona dissection • Sub-zonal insemination • Zona pellucida • Micromanipulation of sperm • Sperm injection in IVF

History and Overview

Company Foundation and Development

In response to the requirement for integrated circuit testing by microelectronic companies such as GEC, Research Instruments (RI) was established by Mike Lee and Vince Grispo in 1964. 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 sub-zonal insemination (SUZI).

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Instrumentation Principles

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 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, a laser-guided setting up device (LASU) launched in 1994, facilitated correct alignment of micropipettes. Another of the 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.

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Fig. 36.1 The IntegraTi

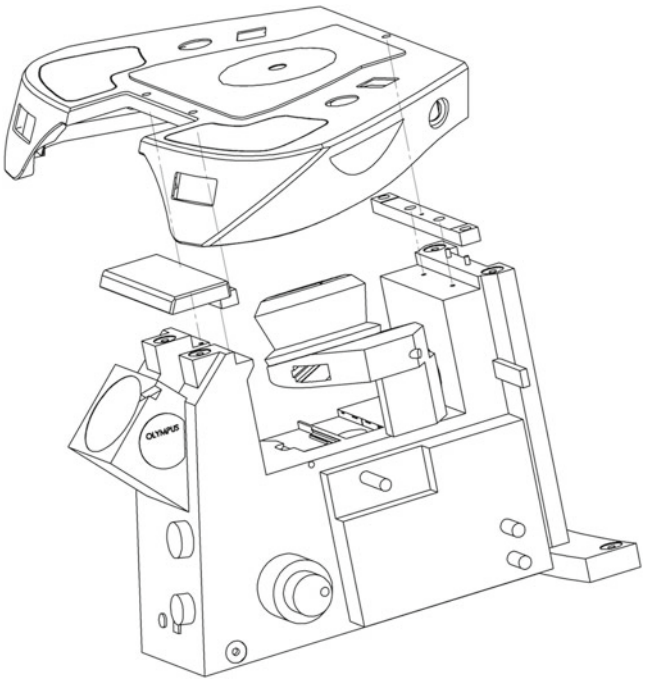

Fig. 36.2 Installation diagram for the IntegraTi to the Olympus IX71

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 introduced semicircular handrests to further improve ergonomics. The current version of the Integra is known as the IntegraTi as it was originally manufactured using titanium for the microscope stage plate. It incorporates a mechanical stage, TDU5000 micromanipulators, touch-screen four-channel independent control of heated plates, a one-touch *home* function, one-touch micropipette angle adjustment and a unique touch-screen digital help menu (Fig. 36.1).

Consistent with original company policy, the TDU5000 micromanipulators remain purely mechanical and are directly controlled, the joysticks providing proportional movement. This policy is based upon 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.

Installation and Set-Up of the IntegraTi™

Installation of the IntegraTi is extremely simple, quick and straightforward as the system comes largely pre-assembled 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 on the company's website (www.research-instruments.com).

The IntegraTi 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. 36.2).

Micromanipulators

The micromanipulators on the IntegraTi incorporate both fine and coarse controls in the 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 TDU5000 also extend downwards but now from within the microscope stage of the Integra. 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.

Tool Holders, Micro-Tool Holders and Micropipettes

RI's PL30 tool holders are supplied with the IntegraTi and are calibrated and actuated using a single screw to enable accurate adjustment to a range of micropipette bend angles, from 15 to 40° (Fig. 36.3). 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 minimising shear stress. A unique feature of the PL30 tool holder is that the tip of the

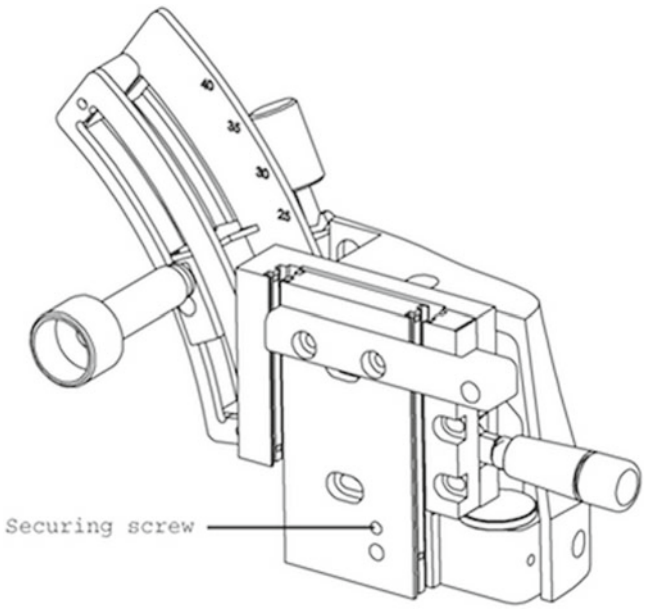

Fig. 36.3 PL30 tool holder

micropipette does not move when the angle is changed, allowing alterations of pipette angle to be made mid-procedure. 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 micro-tool holders can be clipped into place onto the PL30 tool holders (Fig. 36.3). Once fitted to each other, vertical, axial and rotational movement of the micropipette is possible, allowing rapid set-up. When adjusted using an additional objective lens and spacer (supplied by RI), micropipettes can be aligned 14 mm above the microscope stage, minimising the likelihood of damage to them.

A one-off initial set-up of the tool holder is achieved as follows: A scratch is made on the inner surface of a Petri dish using a hypodermic needle, and the dish is placed onto the microscope stage of the IntegraTi. 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 micro-tool holder, and with the vertical movement 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 axis. Drive the MPH micro-tool holder forward until the tip of the micropipette is directly above the objective lens. Rotate the micro-tool holder until the micropipette vertically bisects the objective light path, as viewed with the naked eye, and then tighten the bottom securing screw of the PL30 tool holder. This adjustment effectively sets the detent position of the micro-tool 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 micro-tool holder to obtain a perfectly vertical orientation of the micropipette—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 PL30 tool holder—the scratch 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 minimised by increasing the distance travelled when lowering the vertical lever on the PL30 tool holder. This adjustment is achieved using the small silver thumbwheel located on the side of the PL30 tool holder. 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 pre-implantation genetic diagnosis (PGD), RI also supplies a double tool holder that enables independent movement of both micropipettes.

Microinjectors

The RI screw-actuated syringes (SAS) supplied as standard with the IntegraTi are air-assisted microinjectors that are sometimes referred to as *mushrooms* due to their design. By virtue of their heavy circular base, they are very stable, yet they occupy a relatively small footprint. With an extremely low dead space and a capacity of 10 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. 36.4). This is particularly useful for stabilising 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 that prefer to use an oil microinjector, RI will supply a micrometre-actuated sealed oil syringe (SOS) mounted upon a sturdy, non-slip base.

The SAS microinjectors come supplied with hard polythene tubing for connecting them to the MPH micro-tool 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 micro-tool holder. Once the tubing has been connected and a micropipette fitted to the MPH micro-tool holder, the

Fig. 36.4 SAS air injector with equilibration button

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 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. Secondly, 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 un-pulled shank. At this point, the pressure release button can be pressed down to ensure equilibration of the pressure and so prevent 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.

Heated Stages

The IntegraTi incorporates an independent four-channel temperature control system that is accurately calibrated to within 0.05°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. Two of these channels control two other heated stages, either side of the central insert, towards the front of the microscope stage. These have 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 prep 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 incorporated into the IntegraTi (Fig. 36.1). Since the heated stages come already installed, no set-up other than adjustment of the temperature set points is necessary.

Mechanical Stages

On the IntegraTi, the standard mechanical stages of the *big four* microscope manufacturers have been replaced by a custom-designed built-in XY mechanical stage. It comes as standard and incorporates a stainless steel stage plate. Each turn of the stage control moves the stainless steel stage plate 28 mm in either the *X* or *Y* plane, with 40 mm full travel. Since the mechanical stage is supplied already fitted to the IntegraTi, no set-up is required.

Standard Applications

Zona Drilling and Assisted Hatching

Zona drilling is the technique of creating a hole in the ZP surrounding the oocyte or pre-implantation embryo, originally described in 1986 [2]. The most widely used application of zona drilling in assisted reproduction, termed *assisted hatching*, is based upon the assumption that the blastocyst will more readily *hatch* 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 an inability of blastocysts to *hatch* [3]. Various means have been employed to achieve *assisted hatching* including the use of acids, enzymes and lasers to dissolve the ZP, no single method proving universally better 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 upon 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 PGD.

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 employed to physically cut a slit-like hole in the ZP while holding the oocyte firmly onto a holding micropipette.

Sub-zonal 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 sub-zonal perivitelline space [7, 8]. Originally termed *microinjection sperm transfer* (MIST), the technique later became known as SUZI. Once the manufacture of fine, sharp microinjection pipettes had been perfected, PZD became redundant for the purposes of SUZI.

Intracytoplasmic Sperm Injection

The technique known as 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 fertilisation of the oocyte resulted in its rapid replacement of the SUZI technique. Successful application of ICSI depends upon an appreciation that it must mimic the latter stages of fertilisation that occur in vivo [10]. As with gamete fusion, the sperm plasmalemma and oolemma have to be temporarily broken. 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.

Oocyte, Embryo and Blastocyst Biopsy

Following zona drilling, cells can be removed from the oocyte, early cleavage embryo and blastocyst as biopsy material for the purpose of PGD, 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 PGD, so methods have been developed to perfect PB biopsy. However, since post-zygotic aneuploidy is also possible, although less common, blastomere biopsy of early cleavage embryos at the 8-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 7-cell or 8-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 zona drilling and then to use a laser to separate the extruded trophoblast.

Troubleshooting

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 to adjust the pitch angle to a steeper position.

If the micropipette fails to move smoothly in either the X or Y planes, 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. 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 midpoint by rotating it in the opposite direction in order to free the locked movement. If the fine or coarse control levers are too stiff/loose, the most likely reason for this is that the ball joint is out of adjustment. The remedy for this is to loosen/tighten the screws in the plate that retains the ball joint in place using the appropriately sized hexagonal wrench supplied with the IntegraTi.

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 above in the installation section on microinjectors.

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 micro-tool 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, replace the O-ring inside the barrel and lubricate the O-ring using the special lubricant supplied by RI.

Heated Stages

If the touch-screen display shows a '?' message, the most likely reason for this is a malfunction of one of the heated plates. There is no remedy for this, other than repair or replacement by RI.

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Eppendorf Micromanipulator: Setup and Operation of Electronic Micromanipulators

37

Ehab Abu-Marar and Safa Al-Hasani

Abstract

Eppendorf AG, Hamburg, Germany, has created many devices and tools that are now considered breakthroughs in the field of laboratory applications. The importance of micromanipulators appeared before assisted reproductive technology (ART) proved its effectiveness. In the 1960s, different groups were experimenting with fertility in animal models. Hiramoto found that microinjection of spermatozoa into unfertilized sea urchin oocytes did not induce activation of the oocyte or condensation of the sperm nucleus, whereas others demonstrated the opposite in frog oocytes. Ryuzo Yanagimachi and his group later demonstrated that isolated hamster nuclei could develop into pronuclei after microinjection into homologous eggs, and a similar result was obtained when freeze-dried human spermatozoa were injected into a hamster egg. After the amazing success of ART, scientists began thinking about how to overcome the difficulties faced in fertilizing oocytes.

Keywords

Eppendorf micromanipulator • Intracytoplasmic sperm injection • Sperm swim-up technique • ICSI procedure • Male infertility • Oligozoospermia • Cryptozoospermia • Asthenozoospermia • Teratozoospermia • Globozoospermia • Azoospermia

History and Overview

In 1945 after World War II, two German scientists Dr. Hans Hinz and Dr. Heinrich Netheler founded a small company in Hamburg Eppendorf University Hospital. The demolition of the hospital and destruction of medical equipment during the war necessitated the founding of the company so that the physicians could repair broken devices and develop new ones. The workshop was established under the supervision of both scientists in order to return the hospital to a functioning state. 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. The importance of micromanipulators appeared before assisted reproductive technology (ART) proved its effectiveness. In the 1960s, different groups were experimenting with fertility in animal models. Hiramoto found that microinjection of spermatozoa into unfertilized sea urchin oocytes did not induce activation of the oocyte or condensation of the sperm nucleus, whereas others demonstrated the opposite in frog oocytes. Ryuzo Yanagimachi and his group later demonstrated that isolated hamster nuclei could develop into pronuclei after microinjection into homologous eggs, and a similar result was obtained when freeze-dried human spermatozoa were injected into a hamster egg [1]. After the amazing success of ART, scientists began thinking about how to

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overcome the difficulties faced in fertilizing oocytes. At that time, conventional IVF gave unsatisfactory results. In those days, conventional IVF produced unsatisfactory results in a number of cases. In some of those cases the number of progressively motile spermatozoa with normal morphology was lower than the desired threshold and more assistance was needed for the spermatozoa to reach the ooplasma and for fertilization to take place.

All the efforts were aimed at embracing these cases of limited number of progressively motile sperm with normal morphology. That goal was attained after bypassing or penetrating the barriers which prevent the fertilization (like zona pellucida) and was achieved by partial zona dissection (PZD) and subzonal insemination (SUZI). After that, fertilization, pregnancy, and birth were reported [2–4]. Following these trials, intracytoplasmic sperm injection (ICSI) was adopted by many and showed its effectiveness in 1992 [5]. A micromanipulator's main usage in ART was for assisted fertilization, but many other procedures could be performed with it, especially operations requiring proportional movement like ICSI which became successful worldwide.

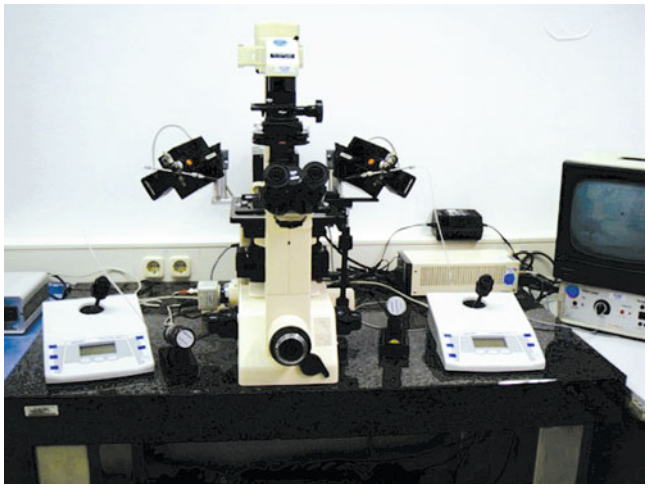

Fig. 37.1 The Eppendorf micromanipulator is composed of three main components: motor module unit, control board, and main power supply

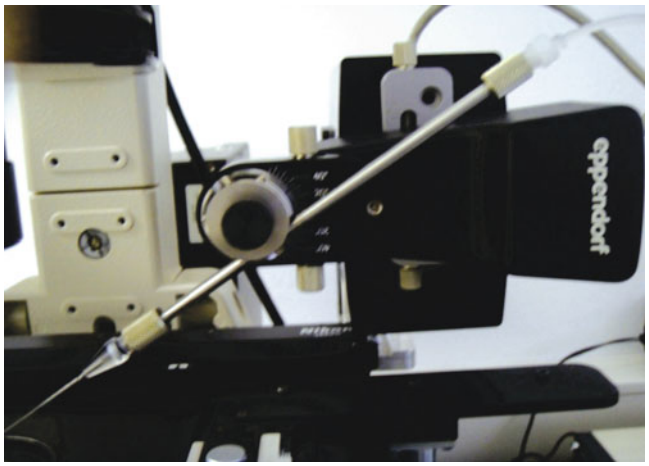

Fig. 37.2 Mounted motor module with attached capillary holder

Description

Components

The Eppendorf micromanipulator (Fig. 37.1) is composed of three main components: motor module unit, control board, and main power supply.

Motor Module Unit

The motor module unit can be fixed on any microscope on any side, but for the best results, especially for ICSI procedures or any cell surgery, the motor modules are mounted via a microscope-specific adapter on both sides of the microscope.

The motor module consists of two main parts attached to each other through a wire and a straight guide fixed with a screw. One part is for *Y* and *Z* axis movements (Y/Z module) and the other one serves *X* axis movements (X module), and this part ends up with X head angle adjuster. Figure 37.2 shows a mounted motor module with attached capillary holder.

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 way and controls position and speed depending on the settings. The joystick can also be disabled by pressing and holding down the joystick top button. The position can be changed to another saved position by pressing the button twice, and clockwise and counterclockwise movements are available. One of the main advantages of the Eppendorf micromanipulator is that it is user friendly even for brand new users. It can save certain positions and limit pipette movements downward and can maintain the same surface level as the Petri dish. This feature is not found in other brands of micromanipulator and greatly reduces pipette breakage by inexperienced and experienced users.
- The display which illustrates the axis coordinates and the options chosen.
- The multifunctional keypad which gives the desired function when the button is pressed or released and allows multiple positions to be saved (Fig. 37.3).

The control board which has a wheel for radius settings on one side.

Fig. 37.3 Control board of Eppendorf micromanipulator

The underside of the control board which has the connector sockets for the module unit and the main power supply as well as the serial port and the connection for an optional foot switch.

The home button which can save the last position while the user prepares for other functions like changing the Petri dish or preparing more oocytes without losing the last position.

ICSI Procedure

Preparation

Preparation for the ICSI procedure starts by proper selection of patients. Because of the high ICSI success rate, it is widely used even in patients who have not been thoroughly screened.

Here are some cases that we believe might benefit from the ICSI procedure:

- After recurrent failure of conventional IVF treatment, ICSI is advised especially in unexplained fertilization failure.
- In patients who undergo an oncology treatment, as ICSI might enhance the fertilization chances especially for patients having cryopreserved tissue.
- In cases of immunological factors impairing fertility.
- For PGD cases to prevent sperm contamination of the sample.
- For almost all cases of male sub- or infertility like oligozoospermia, cryptozoospermia, asthenozoospermia, teratozoospermia, globozoospermia, azoospermia, and cases of retrograde ejaculation, paraplegic patients after TESE, and CABVD where PESA, TESA, or TESE could be done.

After making sure that the necessary equipment and materials are available, it is important to make sure that the micromanipulator and the work station are ready as well.

Make sure that the work place is comfortable then check that the controls are functioning properly. One of the important advantages of the Eppendorf micromanipulator is that it offers the opportunity of saving in memory certain preferred positions so that they can be recalled at any time. For accurate results, you must make sure that there are no bubbles present. We recommend using a vibration-free work station, to enhance the reliable performance by protecting sensitive instruments and equipment from faulty operation or failure. The heating stage of the microscope should be assessed for suitable temperature before starting the procedure.

After oocyte retrieval, cumulus and corona cells should be dispersed by enzymatic and mechanical methods including incubation for 1 min in Sage medium with about 60 IU hyaluronidase/mL. Then aspiration of the cumulus-corona-oocyte complexes takes place with 250–300 and 200 μm opening pipette respectively. After that, all complexes should be transferred in a 5-mL Falcone tube with 1 mL preequilibrated Sage medium.

Oocyte rinsing comes next in Sage medium after observation under inverted microscope to check for germinal vesicles and polar body presence as well as zona pellucida assessment.

After this, we usually incubate the oocytes in 25 μm microdrops of Sage medium covered by mineral oil at 37°C in an atmosphere of 5% CO_2 in air, and then make the metaphase II oocytes selection for the ICSI procedure.

Semen analysis and selection is performed to make sure that a sufficient number of spermatozoa are available for the ICSI procedure. Ejaculated sperm preparation involves seminal fluid removal by washing using medium and centrifuging twice at 500 × *g* for 5 min, followed by supernatant removal. We then suspend them for the swim-up or mini swim-up method [6]. Others might use a procedure of passing the specimen through 2–3 layers of discontinuous Percoll gradient, ending by centrifugation [7, 8].

Epididymal sperm is recovered by microsurgery then dealt with as the ejaculated ones are. Freshly recovered from the epididymis proximal caput, some sperm could be frozen to avoid surgery in future cycles [9–11]. Testicular spermatozoa were isolated from a testicular biopsy specimen. The tissue was then transferred into a Petri dish with Sage medium and torn into pieces during the heated stage of a stereomicroscope, then removed and medium centrifugation at 300 × *g* for 5 min. The pellet was then resuspended for ICSI [7, 12–15].

Technique Description

The ICSI procedure should be performed with the assistance of two bent needles angled at 30–40 degrees, at 200–400 magnification microscopy at a heated stage. Oocyte fixation is done as it is attached gently but firmly to the holding pipette with the help of negative pressure created by the CellTram Air device and then keeping the polar body at 6 or 12 o'clock, after which the single live spermatozoon is immobilized either by a quick movement of the TransferTip (ICSI) capillary via the tail, or by pressing the tail of the sperm cell against the bottom of the dish, then aspirating it (tail-first) into the injection pipette. Move the spermatozoon along the pipette and bring it to rest at its very tip by rotating the knob of the CellTram vario. The injection pipette containing the spermatozoon is introduced at the 3 o'clock position into the cytoplasm through the zona pellucida. Then the spermatozoon is released to pass into the cytoplasm with the smallest amount of medium [16].

After the procedure is completed, the oocyte washing takes place in 25 μL microdrops of B2 medium in a Petri dish and is then stored at 37°C in an incubator containing 5% CO_2 in air.

Good preparation and technique are important in order to make the ICSI service available to a wide number of laboratories [17]. It is also important to make it more comfortable for patients. For troubleshooting tips and error message remedies, consult the TransferMan NK 2 Operating Manual (Eppendorf).

Future Aspects and Considerations

The following considerations and suggestions are offered to tune and streamline the process with the goal of improving the ICSI procedure:

- Smaller microscope and manipulators could be developed to reduce the space requirement.
- The number of the manipulators per microscope might be increased to facilitate more operations to be performed.
- Development of one complete compatible station which includes the microscope, manipulator, temperature adjusting system, comfortable stool, camera, and monitor will help make the station work more smoothly.
- Forming a team taskforce comprised of representatives of the laboratory, manufacturing company, and physicians to assist in idea exchange and technology advancement.
- Robotic micromanipulation might be invented to make distance work possible and make movements coordinate with each other like human fingers.
- More tactile movements are better and more meticulous for this type of cell surgery.
- Foot pedal functions might help the manual part and decrease the pressure on it.
- A more ergonomically efficient workspace that keeps the operator from having to move when taking new dishes and discarding dishes that are not needed anymore.
- Better flexibility and bigger axis diameter to the device movement.
- Better position for the operator to decrease the stress and effort on the eyes and neck.
- More training sessions on the manipulators to produce more skilled operators.

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The Leica Microsystems' IMSI System

38

Christiane Wittemer, Bruno Laborde, Frederic Ribay, and Stephane Viville

Abstract

The evaluation of the fine morphology of the living spermatozoa through the MSOME technique led to the development of *intracytoplasmic morphologically selected sperm injection* (IMSI). This promising technique requires a highly specialize material and a significant training period. Our long-standing experience in this domain allows us to propose in this chapter a detailed step-by-step protocol of the IMSI procedure, enhanced with personal practical remarks. The IMSI working station described here (Leica AM 6000) results from collaboration with Leica Microsystems.

Keywords

Intracytoplasmic morphologically selected sperm injection • Intracytoplasmic sperm injection • Motile-sperm organelle morphology examination • High magnification • Spermatozoan nuclear vacuole

In 2001, Bartoov and collaborators introduced a new concept for observing spermatozoa called *motile-sperm organelle morphology examination* (MSOME) [1]. This technique allowed examination of the fine nuclear morphology of motile spermatozoa in real time at a magnification of up to ×16,000. The same authors consequently established a new intracytoplasmic sperm injection procedure called intracytoplasmic morphologically selected sperm injection (IMSI) and reported on the benefit of selecting spermatozoa using such a technique [2].

Like many other IVF teams, we were immediately interested in this new technique and its possible implications for the treatment of infertility. However, unlike the original system described by Bartoov, we wanted a practical, easy-to-use system, which allowed a single person both to select spermatozoa at a high magnification level and to inject these sperm into the oocytes, without having to change the Petri dish or the microscope. With this goal, we started a productive collaboration with Leica Microsystems, which resulted in a new user-friendly workstation that we have used successfully since 2005 and which is described in this chapter.

Overall Description of the System

This workstation is comprised of the components shown in Fig. 38.1:

- The Leica DMI6000 B microscope (equipment labelled 1 in Fig. 38.1) is fully motorized for its different functions: the field depth and aperture diaphragms; the condenser S 28 with its slits and Wollaston prism; the stage X/Y movement, fine and fast; the nosepiece with its different

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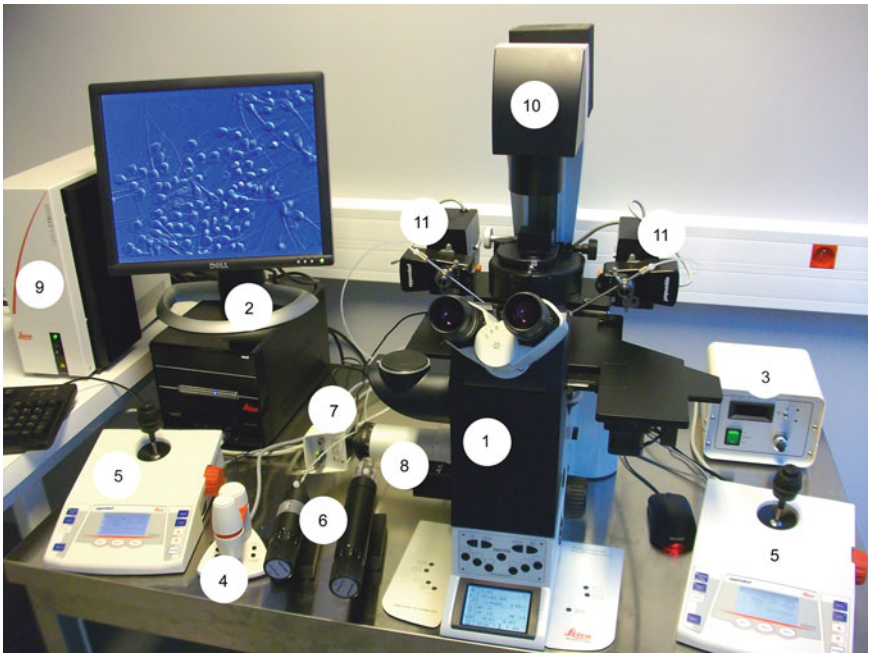

Fig. 38.1 The different components of the LEICA AM6000 working station: (1) Leica DMI6000 B microscope, (2) PC with LAS software, (3) temperature controller, (4) SmartMove, (5) micromanipulators, (6) microinjectors, (7) camera, (8) Vario Zoom, (9) CTR6000 controller, (10) microscope's column, (11) pipettes' motors

objectives (×10 and ×20 dry, ×20 and ×100 immersion oil) and the turret of the Wollaston objective prisms. A magnification changer allows an additional optical magnification of ×1.6. The different contrast methods, bright field, integrated modulation contrast (IMC), and differential interference contrast (DIC), can be activated through the Leica Application Suite software © (LAS) installed on a PC (equipment labelled 2 in Fig. 38.1), which displays all the microscope functions, or by pressing simple knobs placed on the microscope stand. The motorized stage is equipped with a heated insert (diameter 30 mm) connected to a temperature controller (equipment labelled 3 in Fig. 38.1). A remote controller, the SmartMove (equipment labelled 4 in Fig. 38.1), allows the operator to focus and control the stage movements without touching the microscope itself.

- A set of two Leica AM6000 electrical micromanipulators (equipment labelled 5 in Fig. 38.1) (developed in partnership with Eppendorf).
- A set of two Eppendorf microinjectors (equipment labelled 6 in Fig. 38.1): Cell®Tram Oil, for the injection pipette, and Cell® Tram Air, for the holding pipette.
- A digital camera (equipment labelled 7 in Fig. 38.1) (DFC 290) connected to a PC fully controlled by the LAS. A continuous zoom is applied to the camera through the Vario Zoom (equipment labelled 8 in Fig. 38.1). This optical component easily provides magnification on the screen varying from 1,000 to 12,500 times.

The Step-by-Step Protocol of an IMSI Procedure

Preparation of the Workstation

Switching on the Components in a Precise Order

- Switch on the microscope with the CTR6000 controller (equipment labelled 9 in Fig. 38.1) and wait for the complete end of the microscope initialization.
- Use the X-Y controls of the SmartMove to set the motorized heating stage in the centre of the circular hole above the ×10 dry objective.
- First, switch on the right micromanipulator and then the left one.
- Switch on the PC and then launch the LAS Software.

Installation of the Pipettes

- Put the column (equipment labelled 10 in Fig. 38.1) of the microscope to its backward position and the two stands of the pipettes' motors (equipment labelled 11 in Fig. 38.1) on the side in order to have convenient access to place the pipettes.
- Mount the holding pipette (30° angle from Conception Technologies, USA) in the Cell® Tram Air grip head and the injection pipette (30° angle, provided by Humagen, USA) in the Cell® Tram Oil grip head.
- Place the stands of the pipettes' motors to their initial position, and using the manipulators, bring the tip of the pipettes in the bright field above the ×10 dry objective.

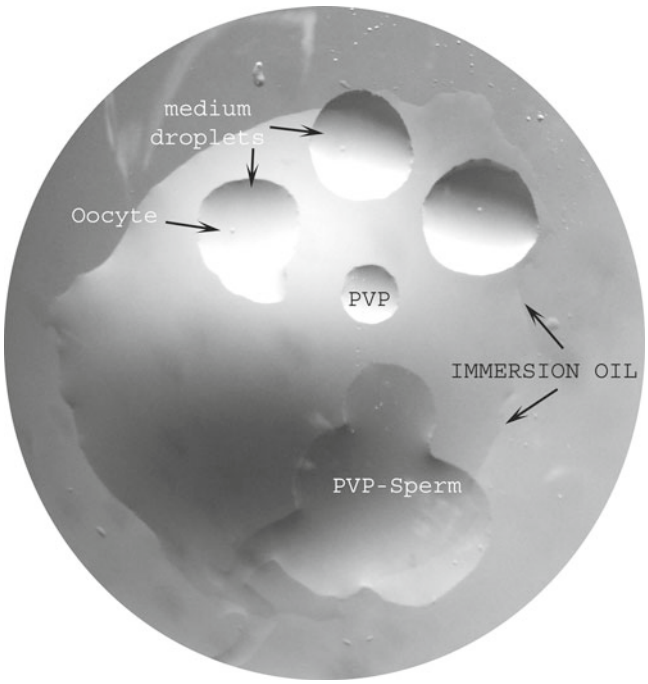

Fig. 38.2 The glass bottom dish with the different drops of medium

- First, adjust the position of the holding pipette until it appears in a horizontal, central and clear position on the screen. Then record this upper position by pressing the button POS1 on the control unit of the micromanipulator until you see P1 for recording the position.
- Adjust and register the high position of the injection pipette as previously described.

Preparation of the Dish (see Fig. 38.2)

- For IMSI, glass bottom dishes are required to obtain a high image quality using immersion objectives. We used WPI—FluoroDish 20090330, 0.17 mm thick, mouse embryo-tested, sterile single use (Gynemed, Germany).
- Turn the dish upside down and cover with immersion oil (Cargille type DF, Formula Code 1261, USA) until reaching a circular working area of 2 cm. Carefully avoid air bubbles.
- Turn the dish again and put it on the overturned lid to avoid contact between the immersion oil and the worktop.
- Several droplets of 3 microliters of FertiCult™Flushing medium with HEPES (FertiPro, Belgium) are placed next to an elongated polyvinylpyrrolidone drop (PVP Clinical Grade, MediCult, Denmark). The sperm suspension obtained after routine preparation through a two-layer density gradient (Sil-Select, FertiPro, Belgium) is deposited at one end of the PVP drop in order to allow the motile sperm to swim up.
- Place one drop of pure PVP between the HEPES medium drops to store the selected spermatozoa (Fig. 38.2).
- The drops are carefully covered with 2 mL of sterile mineral oil (FertiPro, Belgium).
- Before placing the prepared dish on the microscope stage, the immersion objectives ×20 and ×100 are covered with a droplet of immersion oil.
- Place the dish on the microscope stage and mark the 6 o'clock position on the side with a pen for reference.

Practical remark. According to our experience, a sperm concentration between 0.3 and 10 million/mL in the original droplet is recommended to retrieve enough motile sperm in the PVP droplet at a 10,000 magnification level. Due to the glass bottom, the medium droplets are very unsteady and the dish must always be handled very carefully to avoid mixing the drops.

Preliminary Settings

- The PVP-sperm drop is macroscopically placed above the ×20 immersion objective using the SmartMove. The objective comes in contact with the bottom of the dish and is then progressively lowered using the Z fine movement of the SmartMove until the sperm can be clearly seen in the oculars. Focus on the edge of the PVP-sperm drop and register its X and Y positions with the LAS.
- Repeat the same procedure to register the position of the pure PVP drop.
- Keeping the PVP drop focused in the oculars, bring the injection pipette in the observation field by lowering it with the coarse function of the manipulator. When the pipette appears in the field, switch to the fine function to precisely adjust the position. Then record this low position as POS 2 (P2) by using the control unit of the micromanipulator.
- Bring back the pipette to its upper position, and using the LAS, move to the registered position of the PVP-sperm drop.
- Switch to the ×100 immersion objective and DIC contrast and get the focus right. Tune the live streaming on screen by varying the light intensity, contrast and brightness directly on the LAS. Then record this position which can be called back by double-clicking on the icon, which looks like symmetrical triangles reflected over a horizontal line.
- Check the lower position of the injection pipette by pressing the button P2 on the manipulators and adjust the focus. If necessary, record a new lower position by pressing P2 again.
- The LAS allows precise control of the parameters of brightness, contrast, colour, exposure and all camera features.

Practical remark. Even if these preliminary settings are time-consuming, they are extremely important and have to be

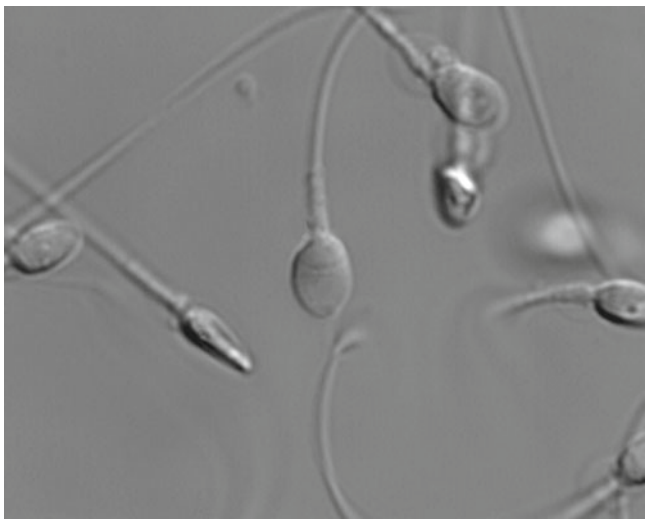

Fig. 38.3 Spermatozoa grade 0: no vacuole

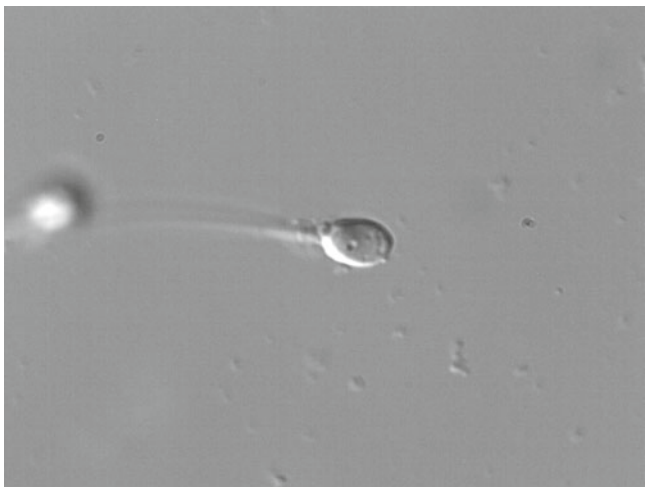

Fig. 38.5 Spermatozoa grade 2: more than two small or one large vacuole

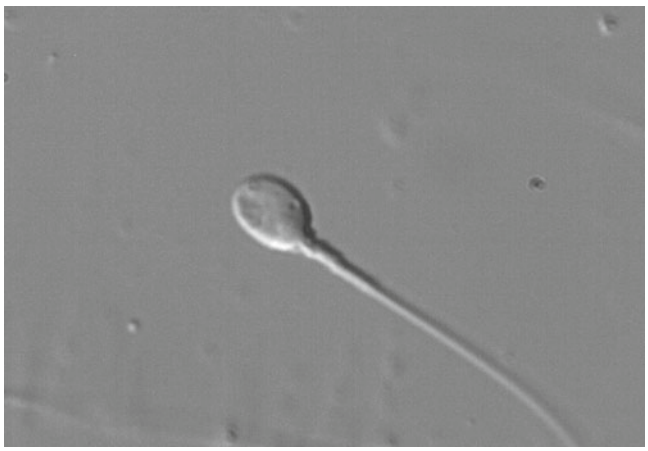

Fig. 38.4 Spermatozoa grade 1: one or two small vacuoles

Fig. 38.6 Spermatozoa grade 3: more than one large vacuole

made carefully for they allow an easier and more efficient sperm selection and IMSI procedure.

Selection of Motile Sperm for IMSI

- A first evaluation of the overall semen quality is performed using the immersion objective ×100. This observation takes about 15 min and evaluates the PVP-sperm drop in order to estimate the best quality of spermatozoa to expect in the sample and to define a minimal threshold of abnormalities which must be expected throughout the selection procedure.
- If possible, spermatozoa that are selected display a normal oval head shape as well as absence of tail defect. Since the influence of the size and number of vacuoles has been well described [3, 4], we classify the spermatozoa into four categories (Figs. 38.3–38.6): grade 0, no vacuole at all; grade 1, one or two small vacuoles; grade 2, more than two small vacuoles or at least one large; and grade 3, several large vacuoles more or less associated with other morphological abnormalities. Small or large vacuoles are defined according to the analysis made by Bartoov and colleagues [2].
- The characterization of the vacuoles is made easier by using the Wollaston prism which gives a three-dimensional aspect.

- A first selection of motile sperm fitting to the minimal threshold defined during the preliminary evaluation is performed at an optical magnification of ×1,600 (objective ×100, magnification changer ×1.6 and ocular ×10).
- The selected sperm is aspirated into the injection needle and stabilized inside it. Still keeping the sperm in view, move the pipette to the drop of pure PVP using the joystick of the micromanipulator.
- The selected sperm is deposited into the PVP drop (position called back on the LAS) and gently moved to the glass bottom by a smooth pressure on the tail, without immobilizing the sperm.
- Collect as many sperm as necessary, usually 1.5 times more than the number of oocytes to be injected.
- A more precise observation of the preselected sperm is then performed by using the Vario Zoom which allows magnification up to ×12,500. By using the Wollaston prism again, some minimal defects can be detected and cytoplasm heterogeneity can be distinguished from vacuoles.
- Each sperm is carefully examined and selected for ICSI or otherwise discarded.

Practical remark. All the described selection procedures are performed at room temperature in order to avoid the negative impact of temperature on sperm fine morphology [5].

It is very important that the selected sperm remains motile until the injection procedure itself. The aspiration into the pipette and the release into the PVP drop must be very gently carried out in order to avoid a premature spermatozoa immobilization which could compromise the fertilization process.

Good management of the drops' position with the LAS and the pipette's position with the micromanipulator allows precise and efficient control of the steps in the sperm selection process without losing any of the precious motile spermatozoa.

The Intracytoplasmic Sperm Injection

Preliminary Settings

- Put the column of the microscope to its backward position and the two stands of the pipettes' motors on the side in order to carefully remove the dish containing the selected spermatozoa. Put the dish on its lid and place it into an incubator at 37°C, long enough to be sure that the medium drops reach the required temperature (about 15 min).
- Switch on the controller of the heating stage in order to perform the ICSI procedure at 37°C (see practical remarks below).
- Switch to the ×20 immersion DIC contrast objective and cover it again with a new droplet of immersion oil.
- After incubation at 37°C, the dish is removed from the incubator, and a maximum of five oocytes are placed into the prepared drops of HEPES medium using a stereomicroscope (see Fig. 38.2).
- The dish is then deposited on the heated stage of the microscope by placing the reference mark at the 6 o'clock position in front of the oculars.
- The column of the microscope and the two stands of the pipettes' motors are moved to their working position.

Practical remark. The temperature displays on the heating controller must be adjusted in advance in order to be sure that the drop itself reaches 37°C. In our case, only when the controller is settled on 45°C is the temperature inside the drops under oil measured at 37°C.

The Injection Procedure

- First, the PVP drop containing the selected sperm must be reached again. Thanks to the reference mark on the side of the dish, the initial recorded position is usually very close to the new position. If necessary, record this new position again on the LAS.
- The position of each drop containing one oocyte is recorded and named on the LAS. The injection procedure can now start.
- In the PVP drop, one sperm is gently removed from the bottom of the dish, immobilized as usual during ICSI, and aspirated tail first into the injection pipette.
- Move to the first oocyte by calling back its registered position on the LAS.
- Bring the holding pipette to its lower position and record it by pressing POS 2 (P2) on the control unit of the micromanipulator. Adjust this position with the fine control.
- Bring the injection pipette to its lower position and check that the selected sperm is still at its tip.
- Aspirate the oocyte with the holding pipette and inject the sperm by the standard procedure.
- Remove the two pipettes to their upper position (P1).
- Repeat these steps for all the oocytes.
- At the end of the injection procedure, put the column of the microscope to its backward position and the two stands of the pipettes' motors on the side and carefully remove the dish containing the injected oocytes.
- Using a stereomicroscope, each injected oocyte is deposited into one 30 mL drop of culture Medium (Global®, LifeGlobal) covered with sterile mineral oil (FertiPro, Belgium) and incubated in 37°C, 5%O_2, 6% CO_2.

Practical remark. The injection procedure is performed using only the ×20 immersion objective. As the insert hole of

the heated stage is small, a maximum of five drops can be positioned around the PVP drop. If more than five oocytes have to be injected, repeat the procedure of injection, using the previous injection drops.

Switch Off the Workstation

- Clean the microscope optics. After each use, an effective cleaning of the immersion objectives is mandatory. Medical wound cotton sticks are required to clean the lens. An alcohol-soaked tissue (avoid lint residues) is used to clean the lens surface and the objectives' body.
- Put the two stands of the pipettes' motors on the side.
- First, switch off the right micromanipulator and then the left one.
- Switch off the LAS Software and then the PC.
- Switch off the Leica DMI6000 B microscope.

Discussion

The development of new tools like the IMSI system Leica AM6000 leads to the discovery of the fine morphology of the living spermatozoa and the presence of the so-called *vacuoles*. The nature of these nuclear vacuoles is unclear, and their number, size and location are extremely different from one sperm to another. Spermatozoa with large vacuoles have a clearly demonstrated detrimental effect on ICSI outcomes [3] probably related to DNA damage [6, 7]. It has been recently suggested that the small vacuoles mostly located in the anterior part of the sperm head could be of acrosomal origin [8].

The selection of spermatozoa without vacuoles appears to be positively associated with pregnancy rates in couples with previous implantation failures ([2, 3, 9], and our own unpublished data) in patients with an elevated degree of DNA fragmented spermatozoa [10] and in patients with severe oligoasthenoteratozoospermia [6]. To evaluate the possible benefit of IMSI compared to ICSI for infertile couples with a moderate oligoasthenoteratozoospermia during their first attempt, a prospective randomized study is still in progress in France.

Apart from IMSI, the MSOME evaluation of sperm represents a much stricter evaluation criteria for sperm morphology than routine methods (WHO or Tygerberg classification) [11, 12] and could be included in the routine laboratory semen analysis and conventional IVF and ICSI procedure.

Conclusion

The Leica IMSI system is a very efficient tool for examining the fine morphology of spermatozoa at a very high magnification level (up to ×12,500) with excellent image quality on the PC screen. Due to good ergonomic design of the components, this workstation allows a single operator to complete the entire IMSI procedure and to easily conduct traditional ICSI without immersion objectives, thanks to the replaceable objectives which can be attached to the nosepiece.

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Automated Robotic Intracytoplasmic Sperm Injection

39

Zhe Lu, Xinyu Liu, Xuping Zhang, Clement Leung, Navid Esfandiari, Robert F. Casper, and Yu Sun

Abstract

Conventionally, oocyte injection has been conducted manually; however, long training, low throughput, and low success rates from poor reproducibility and inconsistency among technicians in manual operations call for the reduction of human involvement and automated injection systems. The past decade has witnessed significant efforts to automate oocyte injection using automation and robotics approaches. Several systems have been reported for robotically assisted oocyte injection. These systems all borrowed the architecture directly from manual operation and automated a few procedures. Commonalities include (1) a holding micropipette for immobilizing an oocyte, (2) an injection micropipette for penetrating the oocyte and depositing materials, and (3) microrobots for positioning the holding and injection micropipettes. Research emphases were placed upon precise microrobotic motion control, development of computer vision algorithms for localization and visual tracking of oocyte and micropipettes, visual servo control of the micropipette, or integration of visual and haptic interfaces. However, there exist several difficulties that prevent these systems or automation techniques from practical use for microinjection. In the state-of-the-art oocyte immobilization process, a robot mimics a human operator to control a holding micropipette to search, hold, and release individual oocyte (~100 μm for mouse and ~150 μm for human).

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The procedures pose tremendous difficulties to a robotic system to achieve a high speed and reliability. Consequently, switching from one oocyte to another has been conducted manually, making robotically assisted oocyte injection cumbersome and time-consuming.

Keywords
Robotic intracytoplasmic sperm injection • Oocyte injection • Methods for robotic intracytoplasmic sperm injection • Microrobots for ICSI • IVF • ICSI

Overview of Robotic Microinjection

Conventionally, oocyte injection has been conducted manually; however, long training, low throughput, and low success rates from poor reproducibility and inconsistency among technicians in manual operations call for the reduction of human involvement and automated injection systems. The past decade has witnessed significant efforts to automate oocyte injection using automation and robotics approaches. Several systems have been reported for robotically assisted oocyte injection [1–5].

These systems all borrowed the architecture directly from manual operation and automated a few procedures. Commonalities include (1) a holding micropipette for immobilizing an oocyte, (2) an injection micropipette for penetrating the oocyte and depositing materials, and (3) microrobots for positioning the holding and injection micropipettes. Research emphases were placed upon precise microrobotic motion control, development of computer vision algorithms for localization and visual tracking of oocyte and micropipettes, visual servo control of the micropipette, or integration of visual and haptic interfaces.

However, there exist several difficulties that prevent these systems or automation techniques from practical use for microinjection. In the state-of-the-art oocyte immobilization process, a robot mimics a human operator to control a holding micropipette to search, hold, and release individual oocyte (~100 μm for mouse and ~150 μm for human). The procedures pose tremendous difficulties to a robotic system to achieve a high speed and reliability. Consequently, switching from one oocyte to another has been conducted manually, making robotically assisted oocyte injection cumbersome and time-consuming.

Furthermore, microinjection usually requires that the polar body be positioned away from the penetration site to avoid polar body damage and increase the chance of further cellular development [6]. In existing systems, oocyte orientation is achieved by repeated releasing and holding or rotating using micropipette until the polar body is located on the 6 or 12 o'clock position. Therefore, orienting oocyte can be a slow and trial-error process. Thus, oocyte orientation is also manually conducted in robotically assisted systems, further decreasing the value of the limited number of automated procedures.

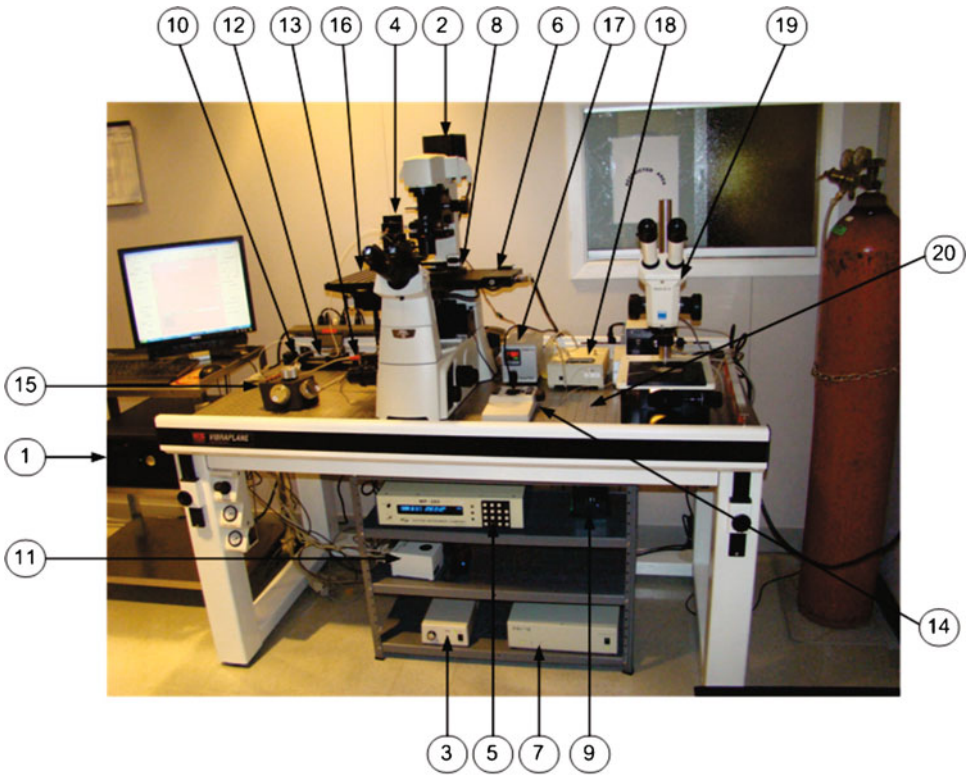
Picking up materials into a micropipette is also a bottleneck in the automation of robotic microinjection due to its complexity. Intracytoplasmic sperm injection (ICSI) is a procedure in which an individual sperm cell is selected, immobilized, and loaded into an ICSI pipette, then injected into an oocyte. There are several challenges in automating sperm manipulation. For example, in sperm tracking, the computer vision algorithm should be robust to variations in sperm motion (e.g., sperm moving in and out of focus; and overlapping of multiple sperm trajectories). In sperm immobilization, the positions of the ICSI pipette and sperm need to be coordinately controlled to properly tap/break sperm tails. Aspiration and depositing single sperm cell also requires the manipulation of sub-picoliter fluids and fast dynamic response of a system.

To overcome the above difficulties in robotically assisted oocyte injection system, we recently developed an automated ICSI system prototype featuring fast oocyte immobilization, automatic oocyte orientation control, automated sperm tracking and immobilization, and oocyte injection. A minimal amount of human involvement remains in the system to ensure system reliability and permits a user to make certain decisions, such as sperm selection. The system is independent of operator skills and immune from human fatigue and/or errors. It has demonstrated a high success rate and a high degree of consistency in mouse zygote injection and is undergoing human ICSI trials. As the first-of-its-kind system, it aims to standardize clinical automated ICSI.

Automated Intracytoplasmic Sperm Injection

System Architecture

The automated ICSI system (Fig. 39.1) consists of a standard inverted microscope (Bright field imaging, 20× objective, Nikon Ti-S), a CMOS camera (601f, Basler), an in-house developed vacuum-based cell holding device for immobilizing multiple oocytes, an in-house developed precision vacuum pump, an in-house developed motorized rotational stage placed on a motorized X–Y translational stage (ProScan™,



1 Host computer
2 Nikon Ti-S microscope
3 Nikon power supply
4 Sutter manipulator
5 Sutter controller
6 Prior motorized stage
7 Prior controller
8 Rotational stage
9 Rotational stage controller
10 Newmark linear stage
11 Newmark controller
12 25 micro-liter syringe
13 CMOS camera
14 Joystick control unit of Prior motorized stage
15 Sutter rotary optical encoder
16 Stand of Sutter manipulator
17 Heating stage controller
18 Vacuum Pump
19 Dissect microscope
20 Vibration isolation table

Fig. 39.1 Automated ICSI system

Prior Scientific Inc.) for oocyte positioning and orientation control, a straight ICSI micropipette (MIC-50–0, Humagen™) connected to a 25-μL glass syringe (Hamilton), filled with mineral oil, and mounted on a linear stage (eTrack, Newmark System Inc.) for computer-controlled sperm aspiration and deposition, a three degrees-of-freedom motorized micromanipulator (MP285, Sutter Inc.) for positioning the ICSI micropipette (45° tilting angle) to diagonally penetrate oocyte, a heating stage (THN-60–10, LINKAM) to maintain oocytes and sperm at 37°, a host computer for controlling multiple motion control devices and processing images in real time, a vibration isolation table (9100 series, KSI), and a dissect microscope (SZX 12, Olympus) for loading oocytes and sperm onto the cell holding device.

Overall Operation Sequence

System operation starts with depositing sperm and oocytes onto the cell holding device under the dissect microscope. The application of a low vacuum immobilizes each oocyte on top of a through-hole on the cell holding device. The culture medium consisting of gamete is then covered with mineral oil.

The cell holding device is then transferred onto the rotational stage on the inverted microscope. Through moving the X–Y translational stage, the first oocyte is positioned at the image center. A straight ICSI micropipette is lowered until the tip of the micropipette roughly appears in the image. The system integrates a vision-based contact detection algorithm

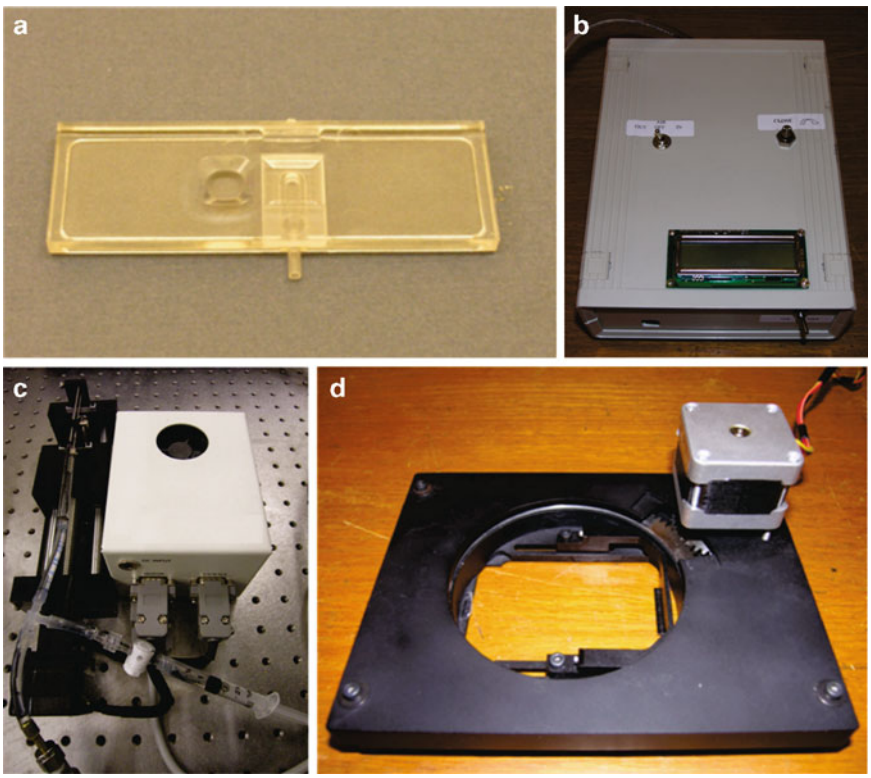


Fig. 39.2 Devices developed for robotic ICSI System: (**a**) cell holding device, (**b**) precision vacuum pump, (**c**) computer-controlled motorized syringe, and (**d**) motorized rotational stage

to automatically determine the vertical position of the micropipette tip and device surface in the oocyte area. The micropipette is then moved automatically to the sperm area to perform contact detection to determine the vertical position of the micropipette tip and device surface in the sperm area. The position of the sperm area is also recorded by the system.

To inject a sperm cell into an oocyte, the user first selects a sperm cell by mouse-clicking the sperm cell head on the monitor of the host computer. The system tracks the motion of the sperm cell and taps its tail for immobilization automatically. Then the user aspirates the sperm cell into the micropipette through the control program interface. When the sperm cell is positioned in the proximity of micropipette opening, an oocyte is automatically brought into the field of view. The user identifies the position of the polar body on the monitor, and if needed, the system automatically rotates the polar body away from the penetration site. The system performs penetration, sperm cell deposition, and micropipette retraction all through computer control. For the next injection, the system moves the sperm area into the field of view for the user to select the next sperm cell for injection. This process is repeated until all the held oocytes are injected.

At the end of ICSI, the cell holding device is taken off the rotational stage and put under the dissect microscope. A low positive pressure is applied to release the held oocytes for collection. The oocytes are then transferred to proper culture medium for further culture.

Devices for Oocyte and Sperm Manipulation

Devices were developed for simultaneously immobilizing multiple oocytes (vs. a conventional holding pipette that immobilizes a single oocyte at a time), orienting an oocyte, and aspirating and depositing a single sperm cell. Related components include a vacuum-based cell holding device with an array of through-holes via which fine vacuum is applied for immobilizing oocytes, a precision vacuum pump, a computer-controlled motorized syringe for sperm aspiration and deposition, and a motorized rotational stage for polar body orientation (Fig. 39.2).

Vacuum-Based Cell Holding Device

Immobilizing multiple oocyte into a regular pattern permits the system to automatically switch from one oocyte to the next, eliminating the need for random oocyte searching. Figure 39.2a shows a vacuum-based cell holding device developed for the automated ICSI system. Evenly spaced through-holes (diameter 70 μm, pitch 500 μm for human oocytes and a diameter of 35 μm for mouse zygotes) are formed on the top layer of the device. The numbers of through-holes are designed to adapt the requirements of mouse, hamster, and human. A vacuum chamber is formed between the top and bottom layer of the device. Upon placing oocytes onto the device, a sucking pressure enables each through-hole to trap a single oocyte. The device proves to be highly effective for rapid, parallel immobilization of many oocytes.

A separate area is integrated on the device to contain sperm. The distance between the sperm area and the oocyte area is ~10 mm.

Precision Vacuum Pump

A portable and stand-alone precision vacuum pump was developed to provide pressure ranging between −2.5 and 2.5 kPa for holding and releasing oocytes (Fig. 39.2b). The pressure system has a resolution of 10 Pa. It consists of two air rotary micropumps. One pump supplies positive pressure, while the other supplies negative pressure. A miniature pressure sensor is integrated to monitor pressure output.

Motorized Syringe

Aspirating and dispensing a single sperm cell requires precision volume control of liquid at varying flow velocities. A computer-controlled linear stage is used to control the motion of the plunger inside a microliter syringe for precisely aspirating a sperm and dispensing an extremely small volume of culture medium when depositing the sperm cell into an oocyte (Fig. 39.2c).

Motorized Rotational Stage

A motorized rotational stage was specifically developed for inverted microscopy use to orient the polar body of an oocyte away from the injection site (Fig. 39.2d). The rotational stage is capable of orienting oocyte with a positioning resolution of 0.08 degree and a rotational speed of 30 degree/s. A clamping mechanism is designed on the rotational stage to fit the cell holding device and make it close enough to the microscope objective. During oocyte orientation, the polar body of oocyte is tracked by a computer vision algorithm. An image-based visual servo controller is used to keep the first target oocyte in the field of view during orientation, when on-line calibration of coordinate transformation between the cell holding device frame and the motorized X–Y stage frame is realized. High-speed oocyte orientation is then achieved on the rest of oocytes in the same batch via coordinate transformation and closed-loop position control.

Methods for Automated ICSI

Vision-Based Contact Detection

A computer vision-based method was developed for the system to detect relative vertical positions of the micropipette tip and cell holding device surface. The fundamental rationale is based on the experimental observation that when contact is established, further vertical motion of the micropipette produces horizontal motion in the image plane. Without requiring the micropipette tip to be in focus, detection starts with the determination of a region of interest and then further detects the contact point using a sub-pixel accuracy method. Experiments demonstrated that the contact detection method is capable of achieving contact detection between a micropipette tip and the cell holding device surface with a sub-micrometer accuracy.

Sperm Cell Tail Tracking and Immobilization

Locating and tracking the sperm cell tail is an important part of sperm immobilization. Thus, the integration of computer vision algorithms for identifying and tracking the sperm cell tail is essential in robotic ICSI. The maximum intensity region (MIR) algorithm for tracking the sperm cell tail has been developed. The algorithm is capable of reliably tracking the sperm cell tail despite the sperm cell tail's fast motion and low contrast under bright-field microscopy.

The sperm cell tail tracking algorithm comprises of three steps. Step 1 exploits the distinctiveness of the sperm cell head to track the position of the sperm cell of interest. To mitigate sperm cell position tracking errors caused by other sperm entering the vicinity of the sperm cell of interest, the sperm cell's movement direction vector is used as a unique identifier that is able to differentiate the sperm cell of interest being tracked from other sperm that are nearby. Step 2 uses the sperm cell head position found in step 1, and the sperm cell's movement direction vector to extrapolate the region in which the sperm cell tail is located. This region, called the sperm tail region of interest (STROI), is approximately from the middle of the sperm cell to the sperm cell tail's tip. Once the STROI is found, the MIR algorithm can be used to locate a position on the sperm cell tail within the STROI.

The MIR algorithm first constructs a flicker image of the STROI by taking the absolute difference between every six consecutive STROI image frames, and summing the resulting differences. The motivation behind this step stems from the fact that an individual frame may not illuminate the structure of the sperm cell tail due to the tail's low contrast and fast motion. However, by utilizing the additive information provided by every six consecutive frames in which the sperm cell is present, the sperm cell tail can be more easily located. After extracting the flicker image, the algorithm locates the region of the flicker image with the highest intensity value. The center point of this region is considered a position on the sperm cell tail.

After the sperm cell tail is located using the MIR algorithm, the sperm cell tail is tracked until its orientation is perpendicular to the micropipette direction. The system then lowers the micropipette and quickly slides over the sperm cell tail and breaks it.

Preliminary Results

The robotic ICSI system was first tested to inject one-cell mouse embryos with phosphate buffered saline (PBS). The injected embryos were cultured inside potassium simplex optimized medium (KSOM) in a 37°C incubator with 5%

Table 39.1 Statistics of non-lysis and blastocyst formation rates of mouse embryos with PBS injection using the robotic system. Injected embryos were cultured in KSOM medium

Experiments	1	2	3	4	5	6	7	Overall
Number of injected embryos	18	18	27	27	50	50	50	240
Number of surviving embryos	18	18	26	27	50	49	49	237
Number of blastocysts	16	15	24	23	46	45	44	213
Non-lysis rate (%)	100	100	96.3	100	100	98	98	98.9 ± 0.6 (mean ± s.e.m.)
Blastocyst formation (%)	88.9	83.3	92.3	85.2	92	91.8	89.8	89 ± 1.3 (mean ± s.e.m.)

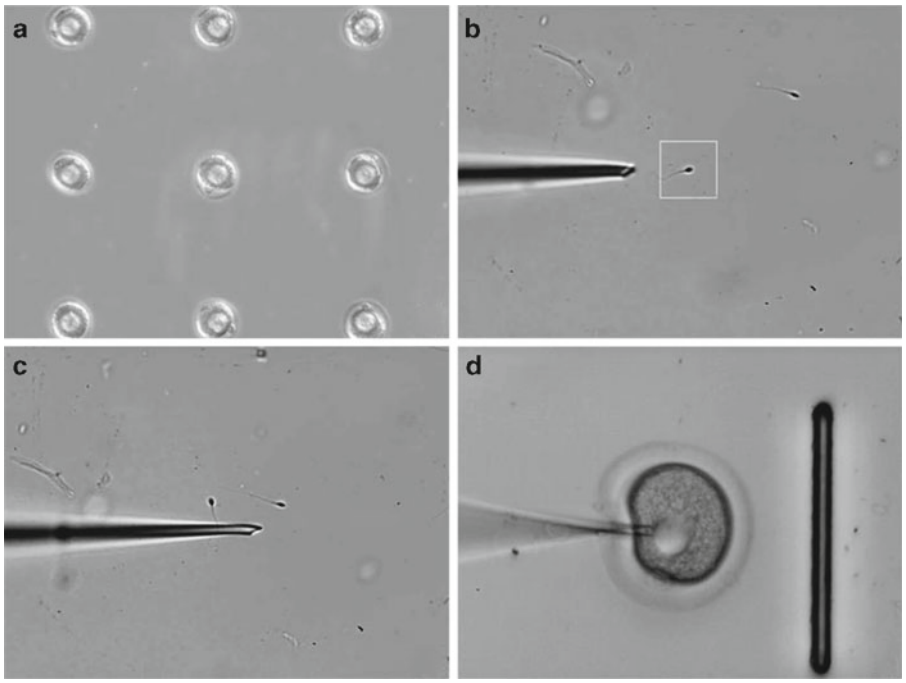


Fig. 39.3 Hamster ICSI: (**a**) an array of immobilized hamster oocytes, (**b**, **c**) a human sperm cell is being tracked and immobilized, and (**d**) a sperm cell is being inserted into a hamster oocyte

CO_2 for 72 h to allow the embryos to develop to the blastocyst stage.

During this phase, two measures were defined [7]: *Survival rate*, which was defined as the ratio of the number of injected embryos without lysis to the total number of injected embryos, essentially representing the frequency of the injection-induced embryo lysis. Based on visual inspection of the 240 embryos shortly after injection, the robotic injection system produced a success rate of 98.9%. *Success rate*, which was defined as the ratio of the number of injected embryos developing into the blastocyst stage to the total number of injected embryos, quantitating the negative impact of microrobotic injection on embryo development. System performance on mouse zygote injection was highly satisfactory, as summarized in Table 39.1.

After the preliminary study on mouse embryos, we performed trials on 1,000 sperm from a healthy sperm donor to evaluate the immobilization success rate of the automated system. The sperm motility was lowered in a viscous culture medium (SpermCatch). The system achieved a sperm cell tail visual tracking success rate of 96%, a sperm cell immobilization success rate of 88.2%, and an average time of 2–3 s per immobilization.

Patient trials are planned. The system is presently injecting human sperm into hamster oocytes as a testing model [8] before moving on to clinical ICSI evaluation. Figure 39.3 shows an array of immobilized hamster oocytes (Fig. 39.3a), a human sperm cell being tracked and immobilized (Fig. 39.3b, c), and the sperm cell being inserted into a hamster oocyte (Fig. 39.3d) by the robotic system.

We expect the automated system to produce a high success rate and consistency in human ICSI, which together with other features such as skill independence and short learning curve will make the system a useful tool for clinical ICSI.

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Oocyte Treatment and Preparation for Microinjection

40

Thomas Ebner

Abstract

It must be emphasized in advance that two major problems must be solved in preparation for intracytoplasmic sperm injection (ICSI). First of all, it has to be ensured that temperature is constantly kept at physiological ranges (approximately 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 setup 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 h 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. 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.

Keywords

Intracytoplasmic sperm injection • Cumulus-oocyte complex • Permeabilization of sperm membrane • Corona radiata • Oocyte denudation • Piezoelectric manipulation of the motile sperm • Immotile sperm

It must be emphasized in advance that two major problems must be solved in preparation for intracytoplasmic sperm injection (ICSI). First of all, it has to be ensured that temperature is constantly kept at physiological ranges (approximately 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 setup 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 h [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,

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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.

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].

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 needs 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.

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 [8]. For reducing the theoretical risk of harming the oocyte, even further incubation time could be shortened (30 s) 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 min (P. Vanderzwalmen, personal communication). Alternatively, plant (Coronase™, Bio-Media, Boussens, France) or recombinant human products (ICSI Cumulase™, Origio, Måløv, Denmark) could be applied [9]. 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 [10]. 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 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.

Catching 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

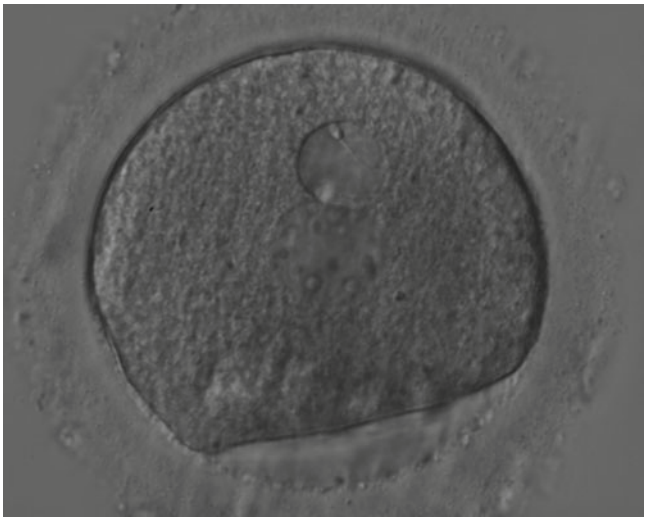

Fig. 40.1 Artificially created vacuole (V) after ICSI (day 1) resulting in a monopronuclear (Pn) zygote. Please note that sperm is located within the vacuole

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 [11]. 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 headfirst 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 [12], there is a much higher risk of artificially creating vacuoles at zygote stage [13] since the larger volume (as compared to sperm headfirst ICSI) of medium entering the oocyte frequently becomes encapsulated (Fig. 40.1).

For these reasons, most embryologists regularly use a viscous water-soluble polymer (PVP) of the monomer *N*-vinylpyrrolidone 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 market, SpermSlow™ (Origio, Måløv, Denmark) and SpermCatch™ (Nidacon, Mölndal, Sweden). Both are based on the finding that a naturally occurring major component of the cumulus cell matrix called hyaluronate (polymeric chain of glycosaminoglycans) slows down the movement of spermatozoa and could act as a natural alternative to PVP [14]. Hyaluronate has a relatively high negative charge and a high hydration capacity which allow 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 [15]. 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 (approximately 1%).

Immobilization of the Spermatozoon

Once the spermatozoon is caught, it appears necessary to immobilize it prior to injection [16]. 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 [17].

Sperm immobilization is usually performed toward the end of the tail (back half); however, permeabilizing alternative sites is also possible. Yong et al. [18] successfully damaged the head membrane of porcine spermatozoa. This is also possible in the human since sperm chromatin is tightly complexed to protamines (approximately 85%) and histones (approximately 15%) and further stabilized by the formation of intramolecular-intermolecular disulphide cross-links between the cysteine residues of the protamine molecules [19]. Thus, any suggested mechanical harm to sperm DNA is only of theoretical nature.

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 [16]. This is in line with the work of Palermo et al. [20] 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 has 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 [16].

Montag et al. [21] introduced laser-assisted permeabilization of the sperm membrane into the field of assisted reproduction. Our study group [22, 23] 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 [24]. The same authors [25] 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.

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 [26]. Not only should embryologists accurately evaluate normal sperm morphology [27, 28] 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. [29] reported that a hyaluronate receptor is expressed in mature spermatozoa only after plasma membrane remodeling 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 ICSI as a convergence to a more physiological fertilization [15, 30, 31]. 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 [15], whereas zygote score was unaffected [30].

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 min 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 [32]. 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 [32].

In practice, it has been suggested [32] 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 a DNA strand break-free from a given processed sperm sample. Recently, our study group (unpublished data, submitted) evaluated the efficiency of a particular sperm selection chamber (Zech-Selector™, 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 Zech-Selector™ strictly separates spermatozoa according to their motility/velocity without exposure to centrifugation stress [33], 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.

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, testicular sperm extraction [TSE] material). In this particular case, embryologists have to be aware that it is of serious consequence if they cannot distinguish between immotile and nonviable 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 [34]. 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 [35, 36]. 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 [37], 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 [38]. Pentoxifylline and other caffeine derivates such as theophylline (Spermmobil™, Gynemed, Lensahn, Germany) are inhibitors of phosphodiesterase activity, which enhance motility in spermatozoa. They show maximum activity after 10 min and an activity phase of less than 2 h [39]. Because of this immediate and short-term effect, direct addition into the droplet containing the immotile sperms is recommended.

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 [40]. 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 [41]. 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 [42] 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. [43] 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 chapter, 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 take place at the site of sperm deposition [44], any deviation from this optimal place, e.g., close to the periphery of the egg, would result in suboptimal pronuclear formation (Fig. 40.2). This scenario is most likely associated with cleavage anomalies or developmental arrest [45].

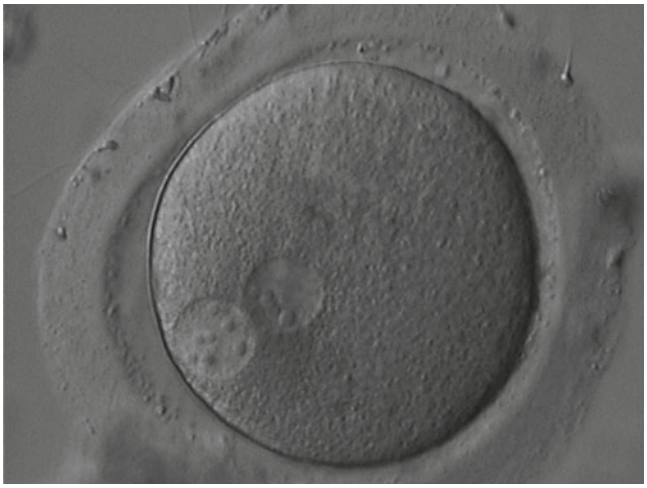

Fig. 40.2 Suboptimal pronuclear formation close to the periphery of the oocyte

Intracytoplasmic Sperm Injection Failure

Since ICSI 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 [46]. The main problems found were as follows: (1) spermatozoon remained attached to the ICSI pipette while being released, (2) insufficient immobilization of the spermatozoon (as assessed by subsequent movement of the tail after injection), (3) rejection of spermatozoon into perivitelline space after ICSI (as assessed by the sperm's tail protruding out of the oolemma/zona pellucida), and (4) difficult breakage of oolemma. None of the above mentioned deviations from a presumed optimal injection procedure is 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 [20], namely, sudden breakage without any invagination during injection and difficult breakage characterized by delayed rupture of the oolemma.

It has been shown that additional manipulation in MII-oocytes showing difficult oolemma breakage may cause an increase in degeneration rate [47]. In order to avoid this scenario, a modified injection technique has been suggested [41] combining a pressing and a sucking phase, thus keeping oocyte survival rate at an adequate level.

Laser-Assisted ICSI

To overcome this high risk for degeneration in such oocytes, an alternative laser-assisted ICSI has recently been suggested [48] and successfully applied in patients with diminished oocyte survival in previous cycles [48, 49]. 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 [50].

However, none of the above mentioned studies took into account a major problem of laser-assisted ICSI, namely, the impossibility to localize the laser-generated hole at later developmental stages [50]. 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 [51], an additional opening is unintentionally created which might impair the hatching process per se and/or result in monozygotic twinning [52].

In order to avoid this possible dilemma, Moser et al. [53] decided not to perform ICSI through a relatively small opening but through a zona pellucida area on which laser zona thinning [54] 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.

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 [55]; 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 et al. [56] reported a modified ICSI 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 [57] which is based on the hypothetical accumulation of high-polarized mitochondria, e.g., showing a high inner mitochondrial membrane potential [58], 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 [59]. In 17 cases of complete fertilization failure after ICSI, we [57] 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 [60].

More recently, an additional modified ICSI technique has been suggested [61], 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 (CULT-aktiv™, Gynemed, Lensahn, Germany) can be recommended in such patients [62]. 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 practice, immediately after ICSI (since the ionophore might alter the constitution of the zona), injected oocytes are incubated in a bath of ionophore (approximately 15 min), whereafter the ionophore has to be removed by carefully washing the ova several times.

Conclusion

ICSI is undoubtedly one of the most severe manipulation techniques in IVF laboratories. Not only that it requires 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 [46, 63]. It is a prerequisite to use ICSI pipettes of standardized quality which 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 are 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 theophylline, 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.

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Livestock Production via Micromanipulation

41

Akira Onishi and Anthony C.F. Perry

Abstract

The use of micromanipulation techniques in the production of livestock mammals, focusing on intracytoplasmic sperm injection (ICSI) and the pig, *Sus scrofa*, is discussed in this chapter. ICSI is a powerful method for assisted fertilization. It is typically employed in cases in which semen characteristics are insufficient for conventional in vitro fertilization (IVF). Unlike IVF, ICSI mechanically delivers the sperm deep inside the egg cytoplasm by injection through a micropipette.

Keywords

Intracytoplasmic sperm injection • Mammalian ICSI • Transgenesis • Xenograft-ICSI technique • Micromanipulation livestock genome

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.

The Application of Intracytoplasmic Sperm Injection

The potential of ICSI in the conservation of genetic resources, transgenesis, and animal production using sex-sorted spermatozoa have 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

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Table 41.1 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					

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. 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 41.1). Nevertheless, IVM oocytes support development to term at comparable rates, making them considerably more cost-effective.

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 41.1), 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 41.1). 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.

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 41.1), 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.

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. 41.1). 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.

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

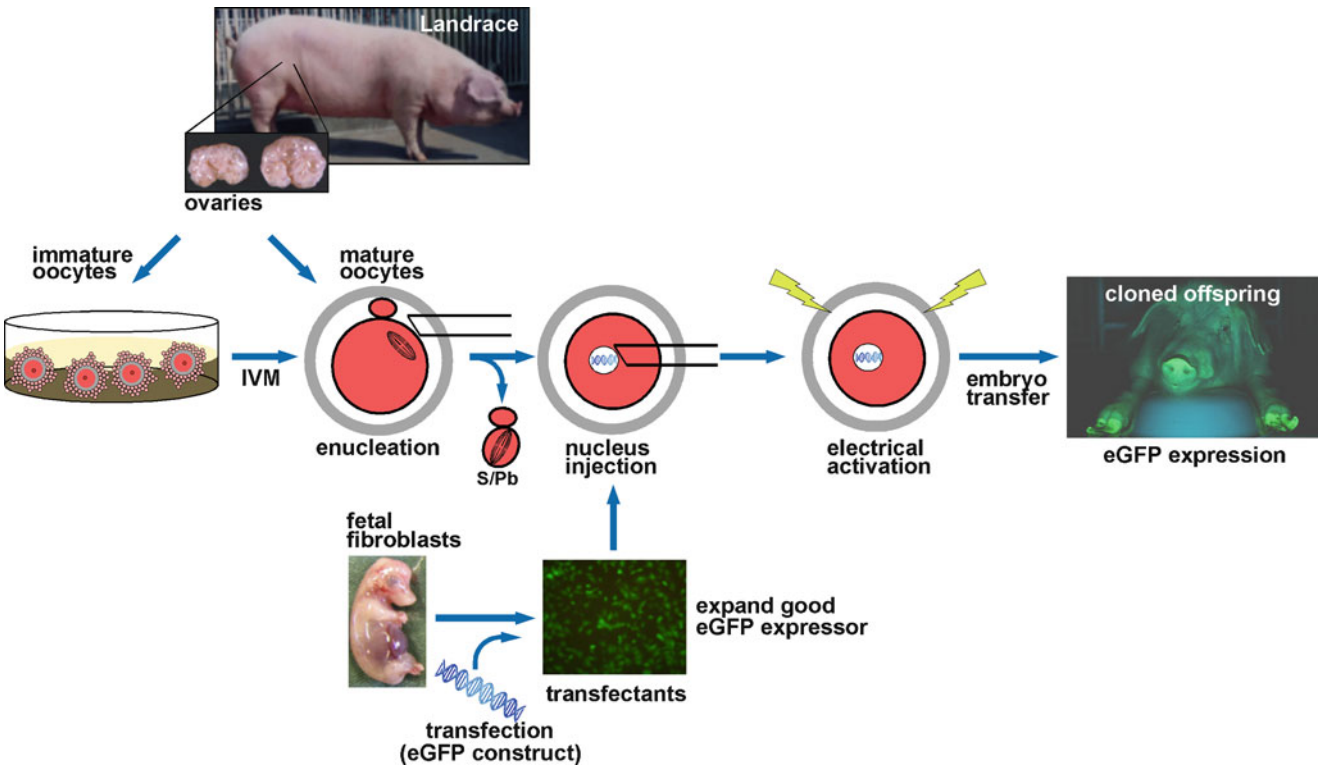

Fig. 41.1 Production of the enhanced green fluorescent protein (eGFP) transgenic pigs by somatic cell nuclear transfer. Fetal fibroblasts were transfected, and cells expressing high levels of eGFP were selected for use as nucleus donors. This method results in cloned pigs that almost always exhibit broad, high-level eGFP expression. *S/Pb* spindle/first polar body

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].

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 six piglets from two 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.

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Part VIII

Embryo Evaluation, Grading, and Assisted Hatching

Pronuclear Scoring in Human In Vitro Fertilization

42

Lynette Scott

Abstract

Historically, the introduction of pronuclear scoring was observational and from two different perspectives, even if the morphological features score resulted in the same outcome and conclusions. Pronuclear scoring involves descriptions of the nucleolar precursor bodies (NPBs) which are clearly visible spherical structures observed in the nuclei of the fertilized oocyte, or for that matter, in the nuclei of any actively dividing cells. The two scoring systems looked at the same parameters of the NPBs, namely, their alignment, number, and relative size in the newly formed male and female nucleus. These parameters were shown to be predictive of continued development and resultant outcome. In the 1999 model from Tesarik et al., the NPBs and their pattern were used as an indicator of sperm decondensation and function during ICSI cases. The model of Scott et al. described patterns of NPBs in the fertilized oocyte and how these changed, how they were related to a halo seen in the oocyte and the dynamic nature of fertilization, and how the patterns correlated with continued embryo viability. Pronuclear scoring is descriptive and based on NPB and nuclear morphology and accomplished at the time of fertilization check, which should be between 16 and 18 h postinsemination. Although descriptive, the patterns have a biological basis and are predictive of development based on cell cycle and structure and function of the nucleus and nucleolus.

Keywords

Pronuclear scoring • Nucleolar precursor bodies • Pronuclear/zygote scoring • Sperm decondensation • Intracytoplasmic sperm injection • Nucleolar organizing region

Historically, the introduction of pronuclear scoring was observational and from two different perspectives, even if the morphological features score resulted in the same outcome and conclusions. Pronuclear scoring involves descriptions of the nucleolar precursor bodies (NPBs) which are clearly visible spherical structures observed in the nuclei of the fertilized oocyte, or for that matter, in the nuclei of any actively dividing cells. The two scoring systems [1, 2] looked at the same parameters of the NPBs, namely, their alignment, number, and relative size in the newly formed male and female nucleus. These parameters were shown to be predictive of continued development and resultant outcome. In the 1999 model from Tesarik et al. [2], the NPBs and their pattern were used as an indicator of sperm decondensation and function during intracytoplasmic sperm injection (ICSI) cases. The model of Scott et al. [1] described patterns of NPBs in the fertilized oocyte and how these changed, how they were related to a halo seen in the oocyte and the dynamic nature of fertilization, and how the patterns correlated with continued embryo viability.

Pronuclear scoring is descriptive and based on NPB and nuclear morphology and accomplished at the time of fertilization check, which should be between 16 and 18 h postinsemination. Although descriptive, the patterns have a

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biological basis and are predictive of development based on cell cycle and structure and function of the nucleus and nucleolus.

Pronuclear/Zygote Scoring

At fertilization, the male and female pronuclei appear on the periphery of the oocyte, early in the process (as early as 6 h after insemination), and rotate into the center by approximately 16–18 h postinsemination, or 56–58 h post-hCG, which is the ideal time for any NPB scoring system [1–6].

In both systems [1, 2], equality of alignment of NPBs in the two nuclei is important, with the numbers of NPBs also being correlated with implantation and development [7]. Where there was equality in both alignment and numbers, there was greater blastocysts development and increased implantation [8–13]. Additionally, the total number of NPBs at the time of scoring is predictive of outcome. There should be between 5 and 7 per nucleus in any mammalian mitotic cell, and for embryos it is also predictive of outcome [7] (Fig. 42.1). From all data, literature, and the consensus meeting [14], an ideal pronuclear score is where there is equality in alignment and numbers between the two nuclei.

The most crucial aspect of pronuclear scoring is the concept of visualizing the oocyte and the nuclei in a three-dimensional state. The oocyte has polarity due to the placement of the spindle, the shape of the spindle, and the attachment of the nuclei to this spindle. This is not in a two-dimensional flat form, but two spheres aligning on a three-dimensional cone structure (the mitotic spindle) within a sphere. Appreciating that the nuclei and NPBs are juxtaposed onto the spindle and that the spindle may lie in any plane between them, and that the chromatin and then chromosomes need to condense onto the spindle, allows an embryologist to mentally rotate the nuclei in order to look at the NPBs' relative to a slice or 2D plane, between the nuclei. In this way, equality and alignment can be easily assigned.

Pronuclear scoring requires the following observations:

- Appearance of the nuclei in a central position is normal with a peripheral position (or directly under the polar bodies) being abnormal, and the nuclei not being in close contact is highly abnormal, resulting in abnormal first mitotic cleavage (Figs. 42.2 and 42.3).
- Relative size of the two nuclei: they should be approximately the same size with no more than a 30% difference in diameter, which is abnormal (Fig. 42.4).
- Equality in both numbers and distribution of NPBs per nucleus with deviations from this being abnormal (Fig. 42.5).
- Presence or absence of a halo, which is an area of clearing of the cytoplasm in the periphery of the oocyte, which is desirable but not necessary (Fig. 42.6).

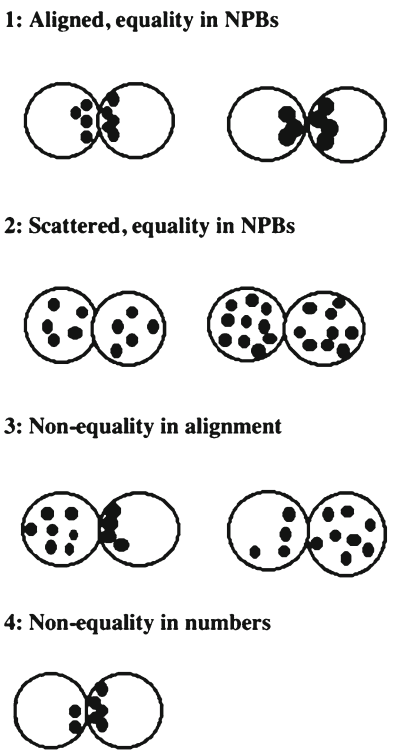

Fig. 42.1 Nucleolar precursor body (NPB) patterns in fertilized oocytes. Patterns 1 and 2 are normal, and 3 and 4 are abnormal

- Major deviations from these patterns such as ghost pronuclei containing no NPBs or pronuclei with one large central NPB: both conditions are highly abnormal and are associated with imprinting errors, abnormal gene activation, and aneuploidy (Figs. 42.7 and 42.8).

Biology of the Nucleus and Nucleolar Precursor Bodies

The question is why are NPBs and their number and alignment important? What is their function in the early embryo, and what is their contribution to an embryo's ability to form a viable pregnancy? Understanding the biology of the NPBs and the structures they function with makes the concept of PN scoring for use in deselecting certain embryos a viable option in an ART laboratory.

The NPBs are part of the nucleoli, which are present in any actively dividing cells. Nucleoli are the organelles in the cell where all proteins are constructed and are the site of ribosomal RNA (rRNA) production [15]. The nucleolus has other functions such as nuclear organization, cell growth through protein assembly, and developmental control [16]. Some growth factors and developmental regulatory proteins are also produced within the nucleoli [17].

Nucleoli are located on the DNA at sites where the genes for rRNA are located (rDNA) and where ribosomal precursors

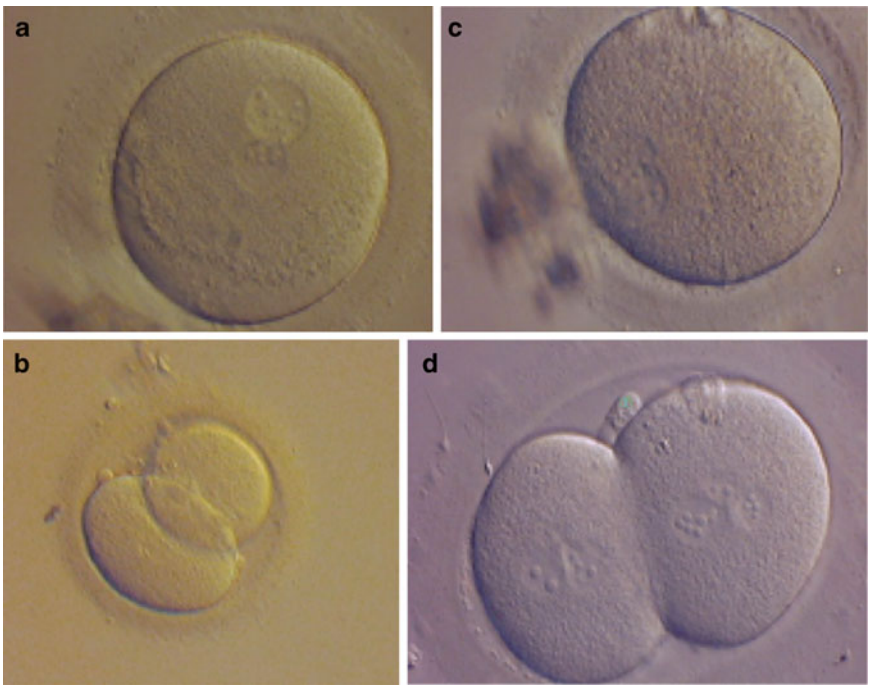

Fig. 42.2 Off-center position of nuclei resulting in abnormal cleavage. (**a**, **b**) Off-center alignment resulting in 2-cell embryo with very unequal cell sizes, which have limited developmental potential. (**c**, **d**) Off-center alignment leading to a 2-cell embryo with severe multinucleation, which results in aneuploidy and abnormal development

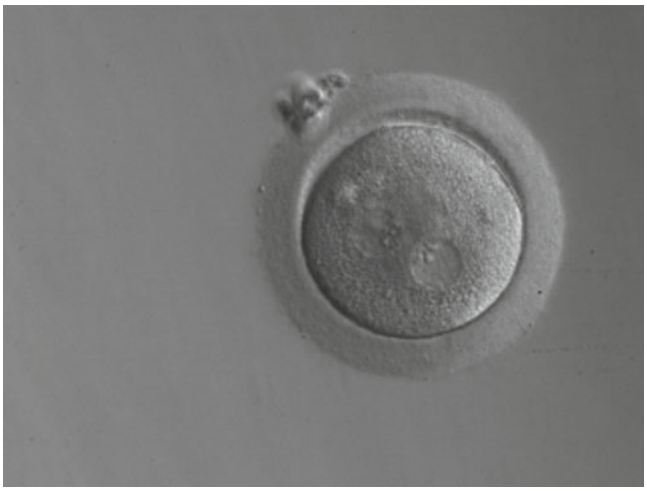

Fig. 42.3 Pronuclear oocyte with nuclei that are not central and juxtaposed

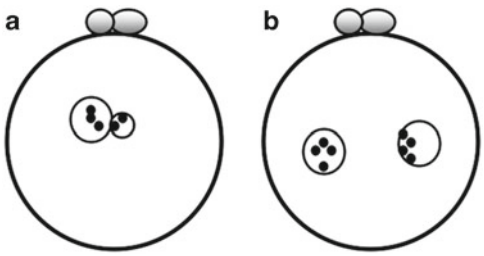

Fig. 42.4 Abnormal alignment or size of nuclei. (**a**) Unequal sizes; (**b**) nuclei not juxtaposed

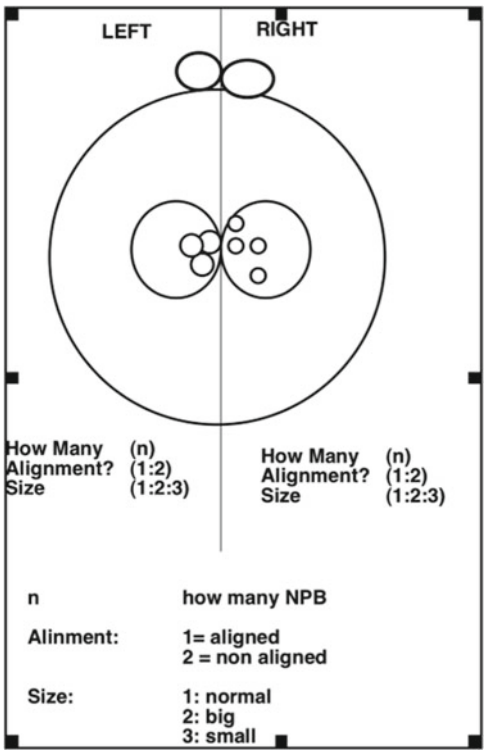

Fig. 42.5 NPB and pronuclear scoring. Representation of scoring

are constructed. These sites are the nucleolar organizing regions (NORs), and they contain many copies of the genes for the major rRNAs, namely, 18S and 28S—rRNA, in tandem repeats [18, 19]. Small rRNA (5S-rRNA) is synthesized outside the nucleolus, as are other nucleolar proteins, such as the factors and enzymes required for transcription and all the precursor units of the ribosomes [19].

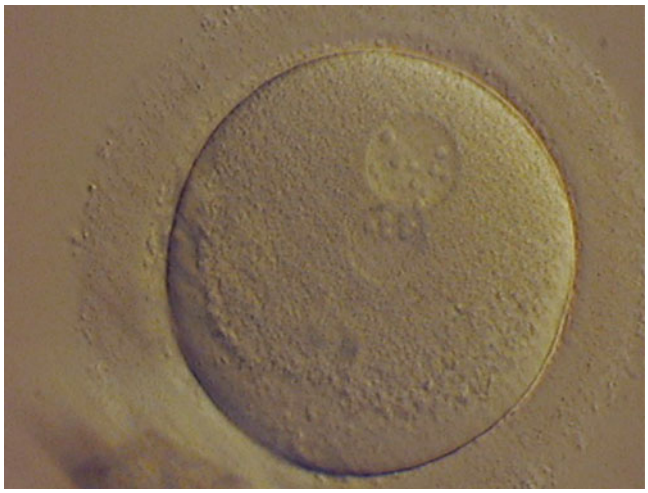

Fig. 42.6 Fertilized oocyte with halo, or clearing of cytoplasm, in the periphery of the cell

The NORs are clustered on the DNA in areas where there is heterochromatin adjacent to rDNA genes. There are only five NOR-bearing chromosomes: 13, 14, 15, 21, and 22; the heterochromatic chromosomes [20] (Fig. 42.9) are also the chromosomes most likely to be abnormal in any aneuploidy screen [21, 22].

Nucleoli in vertebrates contain three functional components: the dense fibrillar component (DFC) that is required for transcription, the fibrillar center (FC) that is surrounded by the DFC, stores inactive transcription factors, and is the center of the nucleolus, and the granular or cytoplasmic component (GC) [18, 19, 23]. The GC usually lies on the periphery of the nucleolus. rDNA transcription and early ribosome biosynthesis occurs in the DFC, and the subsequent steps of forming the mature rRNA, preribosomes occurs in the GC. When cells are not active, the nucleoli are small, and when active, they are large. During development and at each

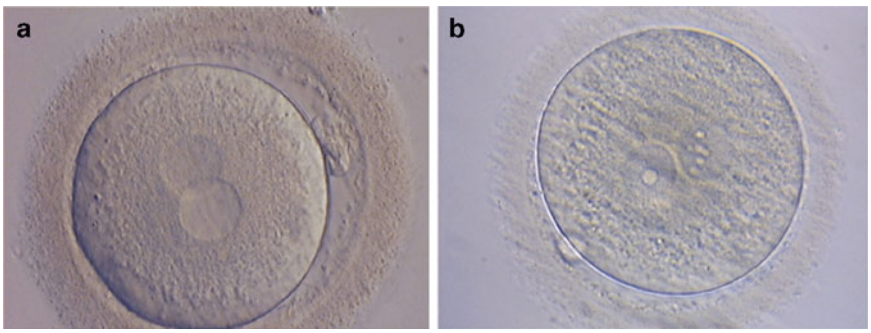

Fig. 42.7 Abnormal fertilized oocytes. (**a**) Ghost nuclei at 16–18 h postinsemination; no NPBs visible, abnormal with risk of imprinting errors. (**b**) Fertilized oocyte presenting with one large central NPB in one nucleus, abnormal sperm nucleus

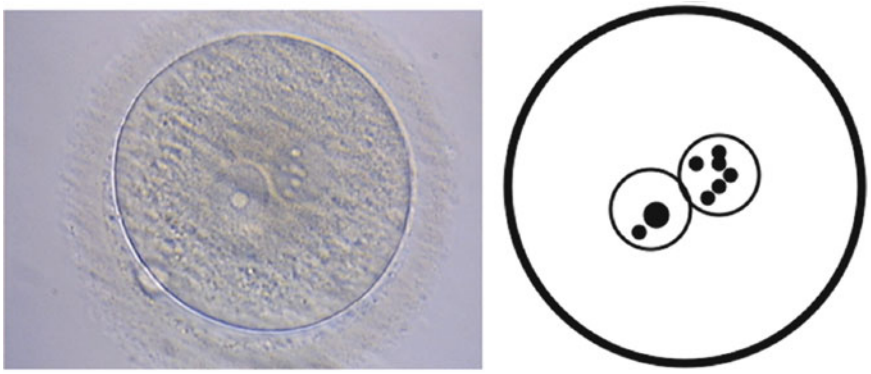

Fig. 42.8 Oocyte with abnormal NPBs in one nucleus, one large central NPB, and the FISH analysis of the resulting 8-cell embryo. The high rates of sex chromosome abnormalities and monosomies result from the abnormal development of the sperm, which fertilized this oocyte

Embryo	XY	13	15	16	17	18	21	22	interpretation
1	0	1	0	0	1	1	2	2	complex
2	X0	1	1	1	1	0	2	1	complex
3	X0	1	2	1	2	1	2	1	complex
4	0	0	2	1	1	1	1	1	complex
5	XXY	2	3	2	1	1	1	1	complex
6	XX	1	1	1	2	2	2	1	complex
7	XY	2	2	2	1	2	1	1	complex

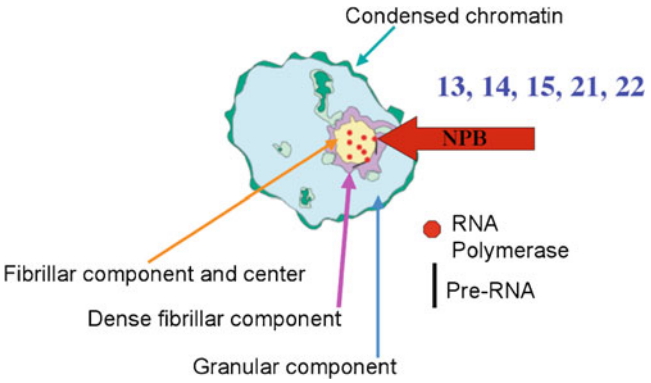

Fig. 42.9 Diagrammatic representation of a nucleolus structure and NPBs and the attachment of chromatin from the five heterochromatic chromosomes onto the spindle

mitotic event, the nucleoli dissemble into their component parts. When they disassemble, the FC region remains and can be visualized in the mitotic nuclei (or pronuclei) as spheres. It is the FC region (the spheres) that is scored or seen during pronuclear scoring; the NPBs are the FC regions of the nucleoli. Since these only reside on the five heterochromatic chromosomes, what is also being seen, indirectly, is the condensation of chromatin from chromosomes 13, 14, 15, 21, and 22 onto the spindle (Fig. 42.9).

Certain cells have a slightly different arrangement, such as Sertoli cells, in which there is only one nucleolus per cell and only one large FC region. Again, this is important in PN scoring for identifying embryos originating from sperm with defects in their RNA and protein assembly apparatus. Fertilized oocytes which present with nuclei in which there is one large central NPB most likely originated from sperm in which the nucleolar apparatus is abnormal, and during sperm maturation, the number of nucleoli has not increased to the normal 5–7. Embryos with this defect do not develop, are frequently chaotic, and are complex abnormal by FISH screening. Pronuclear scoring can deselect these forms of embryos and also allow a biological diagnosis for the infertility and abnormal sperm maturation (Fig. 42.8).

The development of the oocyte from the primordial to mature MII phase results in a huge growth phase, with the oocyte increasing its size 300 times and requiring vast amounts of new protein. Since new protein production requires nucleoli, nucleoli function is very active in oocyte maturation. As the oocyte reaches the final stages of growth, it goes into a resting phase, waiting for the signal for final maturation through the LH surge. At this stage, the nucleoli dissociate (see above) and only reform when the embryonic genome is in place, and the newly formed embryo is growing and requires the creation of new proteins [3–5].

The nucleoli become active during mitosis. As chromatin begins to condense onto the mitotic spindle (in an oocyte at sperm entry), the NPBs will appear to condense onto the spindle. In PN scoring, the lack of symmetry in this condensation is seen as unequal alignment of NPBs, unequal numbers of NPBs, and different sizes which means delayed or fast condensation in one nucleus. This may indicate abnormal or asynchronous karyo- and cytokinesis, which may result in embryos with little developmental potential, hence the use for PN scoring.

Nucleoli are also associated with the process of aging and begin to fragment, resulting in increased numbers of dense bodies in the cells [23]. Thus, inequality in NPBs between nuclei in oocytes will lead to abnormal development, and increased numbers may indicate fragmentation and aging.

Another aspect of nucleoli formation and delays in this, with implications for embryos, is the evidence from nuclear transfer experiments. In these embryos, there is real evidence of delayed embryonic genome activation. This is due to the late onset of functional nucleoli and NPB formation and activation, indicating that NPBs and nucleoli play an important role in embryonic genome activation and cell cycle [25, 26].

In conclusion, the use of pronuclear scoring is an important part of embryo selection in human IVF since it allows for early elimination of embryos that have fundamental flaws in their nuclei and in aspects of protein production and nuclear remodeling during mitosis and general nuclear function. What is scored in PN scoring is part of the cell that is crucial for normal development. PN scoring, performed at the very early stages of embryo development, reflects the health and normality of the gametes, which is essential as a flawed, biologically abnormal gamete is unlikely to produce a normal embryo with full developmental competence.

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Cumulative Morphological Assessment of Embryo Quality

43

Barry Behr and Aparna Hegde

Abstract

It is not inconceivable that in the near future legal, financial, and moral obligations arising from concern over the dangers of multiple pregnancy will compel the limitation of the number of embryos transferred in in vitro fertilization (IVF) patients to just one. Some European and Nordic countries have already introduced mandates to limit the number of embryos that can be replaced while others have issued guidelines and recommendations for the number of oocytes to be inseminated or number of embryos cultured and/or the ability to cryopreserve them. Thus, transferring only one embryo represents the ultimate goal of assisted reproductive technologies (ART), and many studies have been dedicated to the choice of the *right one*. However, an important milestone still to be attained in the practice of ART is the development of a comprehensive reliable and definitive selection protocol for the most viable embryo with maximum implantation potential. Since the inception of IVF, the most widely used criteria for selecting the best embryos for transfer have been based on cell number and morphology. Biochemical methods to assess human gamete and embryo quality have been described by analyzing either follicular fluid or metabolic activity of the embryo. These methods, however, are still very complex, time-consuming, and impractical in most busy ART laboratories. Hence, the assessment of morphology has been, and will remain, the first choice for selection as it is quick and, although not foolproof, has consistently been shown to have some predictive value. With all the morphological criteria described, there is still the limit of a maximum implantation rate, leading to the use of multiple embryo transfers. Also, all morphological methods of selection described rely on a single static observation of the embryos, whereas the embryo itself, after fertilization, is on a very strict set of clocks governing division and initiation of key events from gene activation through compaction and blastulation which is a dynamic process. Each stage of the complex developmental process is dependent upon the successful completion of the previous one. This would imply that optimal evaluation of an embryo's potential would necessitate multiple assessments of the embryo at each of the developmental checkpoints as it makes its transition from a single-celled zygote after fertilization of the oocyte to a blastocyst and from maternal to embryonic genome activation, initiation of protein synthesis, and cell differentiation. Thus, conducting noninvasive evaluation of the preimplantation period in a systematic, cumulative fashion should provide for the most predictive information regarding embryo quality. This chapter will outline the various morphological criteria that

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are currently available for selection within the framework of a continuous model from the oocyte through to the blastocyst. No one selection criteria is better, but if possible a combination of one, two, or even three selection points should lead to more accurate selection. An embryo that does not meet a selection criterion at one point may meet it at another but should be avoided, as it is unlikely that culture can correct an impaired embryo.

Keywords

Embryo grading • Oocyte quality • Pronuclear morphology • Early cleavage • Day 2 scoring • Day 3 scoring • Day 5/6 scoring • Cumulative morphological scoring • Embryo blastocyst scoring • Embryo quality

It is not inconceivable that in the near future legal, financial, and moral obligations arising from concern over the dangers of multiple pregnancy will compel the limitation of the number of embryos transferred in in vitro fertilization (IVF) patients to just one. Some European and Nordic countries have already introduced mandates to limit the number of embryos that can be replaced while others have issued guidelines and recommendations for the number of oocytes to be inseminated or number of embryos cultured and/or the ability to cryopreserve them [1, 2]. Thus, transferring only one embryo represents the ultimate goal of assisted reproductive technologies (ART), and many studies have been dedicated to the choice of the right one [3]. However, an important milestone still to be attained in the practice of ART is the development of a comprehensive, reliable, and definitive selection protocol for the most viable embryo with maximum implantation potential. Since the inception of IVF, the most widely used criteria for selecting the best embryos for transfer have been based on cell number and morphology [4]. Biochemical methods to assess human gamete and embryo quality have been described by analyzing either follicular fluid [5] or metabolic activity of the embryo [6, 7]. These methods, however, are still very complex, time-consuming, and impractical in most busy ART laboratories. Hence, the assessment of morphology has been, and will remain, the first choice for selection as it is quick and, although not foolproof, has consistently been shown to have some predictive value [8].

With all the morphological criteria described, there is still the limit of a maximum implantation rate, leading to the use of multiple embryo transfers. Also, all morphological methods of selection described rely on a single static observation of the embryos, whereas the embryo itself, after fertilization, is on a very strict set of clocks governing division and initiation of key events from gene activation through compaction and blastulation which is a dynamic process [9]. Each stage of the complex developmental process is dependent upon the successful completion of the previous one. This would imply that optimal evaluation of an embryo's potential would necessitate multiple assessments of the embryo at each of the developmental checkpoints as it makes its transition from a single-celled zygote after fertilization of the oocyte to a blastocyst and from maternal to embryonic genome activation, initiation of protein synthesis, and cell differentiation. Thus, conducting noninvasive evaluation of the preimplantation period in a systematic, cumulative fashion should provide for the most predictive information regarding embryo quality. This chapter will outline the various morphological criteria that are currently available for selection within the framework of a continuous model from the oocyte through to the blastocyst. No one selection criteria is better, but if possible a combination of one, two, or even three selection points should lead to more accurate selection [10]. An embryo that does not meet a selection criterion at one point may meet it at another but should be avoided, as it is unlikely that culture can correct an impaired embryo.

Oocyte Quality: Day 0

Mounting evidence that oocyte quality profoundly affects fertilization and subsequent embryo development drives the continued search for reliable predictors of oocyte developmental competence [11]. Five levels of oocyte developmental competence were characterized by Sirard et al. [12], including the ability to resume meiosis, to cleave upon fertilization, to develop into a blastocyst, to induce pregnancy, and to generate a healthy offspring. Cytoplasmic changes accompanying oocyte growth may include messenger RNA (mRNA) transcription, protein translation, and posttranslational modification [13, 14]. These activities are necessary for meiotic maturation [15], activation of the zygotic genome [16], and blastocyst formation [17]. Any dysfunction or dislocation of oocyte components, such as meiotic spindle, cortical granules, or mitochondria, can decrease the oocyte viability and has a crucial impact on embryo development and quality [18–20]. Vlaisavljevic et al. have shown that there is potentially a problem with greater than 50% of all oocytes retrieved in human ART cycles [21]. In a study of two groups of patients with unstimulated cycles who underwent day 2 and day 5 transfers,

Table 43.1 Morphological parameters to assess quality of oocyte

Criterion	Parameters	References
Cumulus–oocyte complex	Compactness and thickness of the cumulus investment, brightness of the cytoplasm	[25–27]
Cytoplasm	Granularity (large or small granules, homogenous or clustering distribution of granules, in center or in the periphery of oocyte), coloration, regions of organelle clustering (vacuoles, endoplasmic reticulum)	[28–30]
Polar body	Shape (round or ovoid), size (large or small), surface (smooth or rough), cytoplasm (intact or fragmented)	[31, 32]
Zona pellucida	Thickness, structure	[33]
Perivitelline space	Size (normal or increased), the presence or absence of grain	[34, 35]
Meiotic spindle	Location and refraction	[36]

From Lasienë et al. [23]—http://medicina.kmu.lt, with permission

respectively, there was no difference in the fertilization rate in the two groups or the achievement of an embryo on day 2. However, only 55% of embryos placed in extended culture grew to the blastocyst stage. This may indicate an intrinsic problem with a large cohort of retrieved oocytes that is exacerbated by ovarian stimulation, or a deficiency in the culture systems used [22]. Overall the implantation rates per embryo transferred were different between day 2 and day 5 (24 vs. 40%), but per oocyte retrieved, there was no difference (12 vs. 14%). Thus, developmental fate of the embryo is largely dictated by the quality of the oocyte [22].

Although the use of cellular and molecular predictors of oocyte quality may be more precise and objective, and no information can be obtained on the day of follicle aspiration because cumulus cells impair evaluation in IVF oocytes, morphological parameters may still provide valuable information for the preselection of oocytes with higher developmental competence. Table 43.1 [23–37] describes the morphological parameters of the oocyte that can be assessed.

Historically, the evaluation of oocyte maturity in conventional IVF has been based on the expansion and radiance of the cumulus–corona complex [38]: Expanded cumulus matrix and a "sun-burst" corona radiata denote preovulatory (metaphase II) oocytes, less-expanded complex denotes intermediate maturity, and the absence of an expanded cumulus might indicate oocyte immaturity (prophase I). However, this method is unreliable in case of disparity of maturation process in oocyte and cumulus as can happen when there is a drop in estradiol levels during controlled hyperstimulation [39].

ICSI facilitates morphological assessment of the oocyte due to the inherent requirement for the removal of cumulus and corona cells. Oocytes retrieved from patients following controlled ovarian hyperstimulation show varying stages of meiotic maturity. Only oocytes at metaphase II are suitable for ICSI, whereas oocytes at metaphase I may only be used if only a few MII oocytes are available as their fertilization rate was found to be rather low [40]. Oocytes at prophase I and giant MII oocytes (which mostly result in digynic triploidy) should be discarded [41, 42]. Disturbances or asynchrony in cytoplasmic [30, 35, 43] and nuclear maturation [31, 44] of the oocyte may result in different morphological abnormalities, respectively. Such disturbances may be caused by reduced blood supply of the follicle during controlled hyperstimulation resulting in oxygen deficiency and reduced viability [45] and abnormal oocyte spindle as well as chromosomal defects [46]. In the presence of such an inherent cytoplasmic defect or disorganization, the formation of polar axes which are associated with morphogenesis throughout pre- and postimplantation stages [47] may be impaired. Also, disproportionate segregation of mitochondria and proteins at the first cleavage division can result in cleavage arrest or a limited implantation potential [7].

It has been recognized that MII oocytes of good morphology should have a clear, moderately granulate cytoplasm, a small perivitelline space, an intact first polar body, and a colorless zona pellucida [31, 34, 48]. However, more than half of all oocytes show at least one morphological abnormality [7].

Cytoplasm and Polar Body

According to the morphological criteria described in Table 43.1, human oocytes are classified as (a) normal oocytes; (b) oocytes with extracytoplasmic abnormalities (dark zona pellucida, large perivitelline space with debris, fragmentation of the polar body, and abnormal consistency of the oolemma and the zona pellucida); (c) oocytes with intracytoplasmic abnormalities (dark or granular cytoplasm and cytoplasmic fragments); (d) oocytes with shape abnormalities; and (e) oocytes with multiple abnormalities [29].

Some studies have revealed that the embryos developed from the oocytes of normal cytoplasmic structure undergo the best uterine implantation [29], while three studies [29, 34, 49] were not able to correlate oocyte morphology with fertilization rate or embryo quality. Serhal et al. [28] described a pregnancy rate of 24% in patients with transfers derived solely from normal oocytes compared to 3% in oocytes with

cytoplasmic abnormalities. Other studies have reported similar results [43]. Kaharman et al. found ongoing pregnancy rates to be poor (12.8%) in patients with granular cytoplasm [30]. A high frequency of aneuploidy was found in many cells of the developing embryo when the oocyte had cytoplasmic abnormalities [29, 30, 48]. A significant decrease in preclinical pregnancy loss has been found in patients in whom none of the replaced embryos was dysmorphic in origin (20 vs. 58.3% in oocytes with multiple dysmorphism) [50] This may be due to higher rate of aneuploidy found in dysmorphic oocytes [30, 51, 52].

Extrusion of first polar body indicating the end of meiotic maturation can easily be examined [7]. An intact first polar body showing a smooth surface was found to be of positive prognostic value in terms of fertilization and embryo quality, as well as implantation and pregnancy rate [31, 53, 54]. Conversely, oocytes with a fragmented first polar body developed worse (55.1%) after fertilization than those with a normal polar body (60.3%) [48]. Navarro et al. [55] reported that the presence of an enlarged polar body is related to poorer rates of fertilization, cleavage, and top-quality embryos. However, identification of first polar body fragmentation did not seem to interfere with ICSI outcomes [32].

Prognostic relevance of first polar body morphology has also been suggested by data for oocytes matured in vitro [56]. It is thought that aging in vivo in MII before aspiration may lead to degeneration of the first polar body and that the resultant overmaturity of such oocytes may further contribute to a diminished developmental potential [31, 44, 53].

Perivitelline Space and Zona Pellucida

Xia et al. [48] found that oocytes with a large perivitelline space developed worse after intracytoplasmic sperm injection (37.5%) than those with normal perivitelline space (60.3%). Oocytes, which had large grains in the perivitelline space, developed worse (59%) after fertilization than those without grains (71.1%) [35]. The thickness of the zona pellucida varies from 10 to 31 μm [57] and is not related to the cytoplasm diameter. The thickness of the zona pellucida influences sperm penetration. Oocytes are fertilized best in vitro when the thickness of the zona pellucida is less than 18.6 μm [23]. Presence of thick zona pellucida (22 μm and thicker) could be an indication for intracytoplasmic sperm injection for infertile patients [58] as it does not have any influence on embryo development following ICSI.

Meiotic Spindle

Meiotic spindle has a significant influence on the correct alignment of chromosomes in the oocyte and their segregation during meiosis. Parameters of meiotic spindle (location and refraction) may be used to determine the quality of oocytes. Nowadays, meiotic spindle can be examined, and its location can be determined by means of polarizing microscopy, avoiding damage to the oocyte as opposed to confocal microscope, which causes damage [23].

The birefringence of meiotic spindle can be studied with the help of a Polscope microscope. It has been estimated that the oocytes with birefringent spindle have higher developmental potential after fertilization in vitro or intracytoplasmic sperm injection than oocytes without birefringent spindle [24, 36, 59, 60]. Moon et al. [36] estimated by Polscope that the location of meiotic spindle could vary in an oocyte. Therefore, it can be damaged when *blind* intracytoplasmic sperm injection is made into human oocytes. However, no relationship was found between deviation of the meiotic spindle from the polar body within oocytes and oocyte developmental competence [24, 36]. Battaglia et al. [61] reported that the number of oocytes with spindle abnormalities (abnormal placement of tubulin) increased with increasing women's age (40 years and older). This abnormality is associated with displacement of one or more chromosomes from the metaphase plate during second meiotic division and thus can cause aneuploidy [61]. The routine use of a Polscope in the IVF lab is challenging as temperature and orientation of the oocyte during the spindle observation can significantly affect the interpretation of the results.

Rienzi et al. derived an MII (metaphase II) oocyte morphological score (MOMS) by identifying relationships among oocyte appearance, fertilization status, PN score, and day 2 embryo quality [62]. Morphological examination of 1,191 MII oocytes showed that presence of vacuoles, abnormal first polar body, and large perivitelline space were related to a lower fertilization rate. Pronuclear morphology was adversely affected by the presence of a large perivitelline space, diffuse cytoplasmic granularity, and/or centrally located granular area. The later characteristic was also found to be negatively related to day 2 embryo quality. According to the odds ratios obtained for each oocyte morphotype, an MII MOMS was calculated (see Table 43.2) [62]. A significant relationship was found between MOMS and female age, female basal FSH, and clinical outcome.

Table 43.2 Metaphase II oocyte morphology scoring system (MOMS)

Extracytoplasmic features	Points
Abnormal polar body	2.0
Large perivitelline space	1.4
Cytoplasmic features	
Granular cytoplasm	1.4
Centrally located granular area	2.7
Vacuoles	2.1

From Reinzi et al. [62], with permission

Zygote Quality Day 1: Pronuclear Scoring Systems

There has been a suspicion that major abnormalities of pronuclear development (i.e., pronuclei of unequal sizes, at a distance or not centrally located within the zygote cytoplasm) are incompatible with normally progressive development [63]. Hence, a number of different noninvasive pronuclear scoring systems have been proposed which rely upon one static observation of simple morphological parameters at the pronuclear stage performed at 16–18 h postinsemination with both standard IVF and ICSI. All pronuclear scoring systems [64–67] 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). Other parameters that may be factored in are polar body morphology and alignment, cytoplasmic morphology (presence or absence of halo), timing of nuclear membrane breakdown, and finally a second embryo evaluation at 25–27 h postinsemination for pronuclear morphology (presence vs. absence of pronuclei) and/or early cell division [63].

The original score 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 [64]. The total score ranges from 7 to 25 with optimal score equal to or above 15. The revised score included only parameters that could be assessed at the time of fertilization assessment (17–18 h postinsemination), classifying the zygotes into five basic groups denoted as *z* score [66] which were later condensed into four basic categories [67]. They also showed an increase of 1.6-fold in implantation rates when the *z*-scoring system was included in embryo selection [67].

Tesarik and Greco, however, claim that it is possible to predict preimplantation development by focusing exclusively on the number, distribution, and synchrony of development of nucleolar precursor bodies (NPBs) in each pronucleus [65, 68]. These authors considered interpronuclear synchrony, evaluated at 12–20 h after IVF/ICSI, to be more important than the actual NPB polarity at the site of pronuclear apposition as they assumed that polarization of nucleoli was not evident from the beginning of pronuclei formation but appeared progressively with time [68]. The optimal synchronized pattern (pattern 0) yielded 37.3% of good-quality embryos compared with only 27.8% of all other patterns.

Ludwig et al. [69], Zollner et al. [70], Senn et al. [71], Kaharman et al. [72], and Gianaroli et al. [73] developed further systems for scoring pronuclear morphology which are all based on a common theme of the value of symmetry in all of the morphological parameters examined.

Testing of the pronuclear morphology scoring systems by various ART facilities reported inconsistent results in their usefulness in predicting embryonic implantation potential [63]. Various studies have shown strong correlation of pronuclear scoring system with blastocyst development potential [66, 67, 74], implantation rates [64–66, 69, 74–78], pregnancy rate [78], and chromosomal status [72, 73, 79–83].

However, Salumets et al. [84], James et al. [85], Jaroudi et al. [86], and Payne et al. [87] failed to show any correlation between zygote score and pregnancy rate. No agreement also currently exists regarding the utility of using the cytoplasmic halo as a marker of embryo quality [88]. Salumets et al. [84], Scott [22], Ebner et al. [7], and Stalf et al. [89] found a positive association, while Zollner et al. [70] found that a halo of extreme dimensions might have a detrimental effect on blastocyst development.

Undoubtedly, pronuclear scoring is a rapid, simple, early, noninvasive selection technique requiring one static observation of simple morphological parameters at 16–18 h postinsemination. However, no standardized scoring system for zygote grading is currently in use. Hence, comparing success rates between laboratories while controlling for embryo quality is a challenge [63]. Pronuclear morphology is also very fluid, and there is considerable biological variation within normal development with different numbers and dimensions of nucleoli being compatible with development. Given the dynamic nature of PN formation [68], including NPB distribution, migration, coalescence, and dissolution, the timing of evaluation is of critical importance. [Zygotes formed from ICSI reveal their PNs approximately 4 h earlier than those formed by routine examination [88, 90].] The scoring is more time-consuming than the cursory assessment of the zygote that is currently done for determination of fertilization. Accurate assessment of the 3-dimensional disposition and number of NPBs is difficult as it requires the visual memorization of the spatial organization of the structures through multiple focal planes, and this memorization must be done rapidly to avoid prolonged exposure of the zygote to light, to temperature, and to pH shifts [88]. Further refinement of the scoring system is needed, and additional features such as the ratio of the NPBs per nucleus [91]. The orientation of the pronuclei relative to the polar bodies [6, 92] may need to be assessed for possible inclusion of the pronuclear scoring systems due to studies suggesting association with outcomes.

Significance of Timing of First Cleavage

When the final steps of oocyte maturity are triggered by LH surge (hCG injection), a clock is set in the oocyte, which will

mark the time since this event all through the development of the preimplantation embryo [9]. Strangely, this time point is seldom used when assessing embryos. Any delay in a developmental event will result in the embryo being out of synchrony with the clock [22]. A 4-cell embryo scored in the morning of day 2 is definitely not the same as one that was scored as 4-cell in the afternoon [6]. Thus, one of the most critical factors in determining selection criteria for embryos is to ascertain strict time points to compare embryos [93]. The first cleavage to the 2-cell stage at 24–27 h after insemination or microinjection has been shown to be one such critical time point for selecting embryos for transfer [94, 95]. Early entry into the first mitotic division accounts for early completion of the final events of fertilization alignment of the pronuclei, alignment of the chromosomes on the metaphase spindle, and finally the first mitotic division. Also, early cleavage is a clearly visible event, whereas pronuclear morphology may vary during the dynamic process of syngamy [68]. Entry into first mitotic division by 22–24 h after insemination has been correlated with increased blastocyst formation [96, 97], increased implantation rates [95, 98–103], increased pregnancy rates [84, 94–96, 102, 103], and presence of a euploid set of chromosomes [100, 104]. Significantly, in the original embryo scoring system described by Scott and Smith [64], embryos that had already cleaved to the 2-cell stage by 25–26 h postinsemination were assigned an additional score of 10. Terriou et al. [105] found that even early first cleavage was strongly associated with good embryo morphology on day one, and multivariate analysis demonstrated that early embryo cleavage and embryo score on day one had strong complementary predictive value for pregnancy.

Interestingly, first cleavage can occur earlier in ICSI zygotes compared to those obtained by IVF [100]. This is because the direct injection of a spermatozoan bypasses most of the fertilization steps and results in a shorter fertilization time [90]. Hence, Lundin et al. [100] suggest that it may be better either to inseminate oocytes earlier or screen IVF zygotes later in order to obtain comparable data. However, until more studies are available on ICSI embryos, checking embryos on day 1 at 22–24 h after insemination can be considered a simple and noninvasive procedure that may help to select embryos in which the time clock is advanced or at least not delayed [22].

Day 2 Scoring: Multinucleation and Blastomere Morphology

During the early years of conventional IVF, oocyte quality could not be estimated, and there were no day 1 scoring systems available [7]. Hence, grading systems for day 2 embryo based on the number (representative of cleavage speed), shape of the blastomeres, and percentage of fragmentation were introduced [7, 38]. The various day 2 scoring systems that have been proposed (see Table 43.3) [3, 4, 38, 88, 106–110] are either exclusive day 2 scores [106, 108, 109] or include grading that can be done on either day 2 or 3 [4] or grading that includes parameters observed on both day 2 and 3 [3]. Van Royen's "top-quality embryo" includes multinucleation on day 2 in addition to day 3 parameters [3]. Though various studies have indicated a positive association of transfer of high-quality embryos as determined by the various scores with improved developmental potential, implantation rate, and pregnancy rate, a major problem with most of these studies was the impossibility of knowing which embryo actually implanted, as the embryo transfers were grouped possibly resulting in contradictory results [7, 111]. However, Guerif et al. [111] cultured more than 4,000 embryos individually in microdrops and sequentially evaluated them from day 1 to day 6, and they found that early cleavage and cell number on day 2 were the most powerful parameters to predict the development of a good morphology blastocyst at day 5. The grading system [111] they used is as follows: Blastomeres were classified into three groups (<4 cells, 4 cells, >4 cells). The degree of fragmentation was expressed as a percentage of the total oocyte volume occupied by anucleate cytoplasmic fragments. The rate of fragmentation was scored (<20% of the volume of embryo, between 20 and 50% of the volume of the embryo, >50% of the volume of the embryo). Embryos with one or more multinucleated blastomeres were excluded from extended embryo culture. Embryos with pattern 0, early cleavage, four regular blastomeres, <20% of fragmentation, and no multinucleated blastomeres were classified as *top quality* [111]. Scott et al. [91] also found that in addition to pronucleate morphology, day 2 morphology of cleaving embryos is stronger positive predictors of implantation than day 3 morphology or the ability to achieve the blastocyst stage of development. Parameters that were most consistently correlated with no delivery were lack of pronuclear symmetry and abnormal day 2 morphology parameters, multinucleation, and uneven cell size (defined as >20% difference in size/volume of the cells [91]).

Multinucleation

Day 2 state of nucleation has proven to be highly significant in terms of fetal development and delivery [91]. Multinucleation is a common phenomenon with the presence of multinucleation reported in 79.4% of all cycles by Van Royen et al. [112] and 74% by Jackson et al. [113]. Significantly, the observed multinucleation is an underestimation of the actual rate as the percentages reported in literature only reflect multinucleation at the interphase stage when the nucleus is visible [112].

Table 43.3 Examples of various scoring systems for embryo selection

References	Name	Day of assessment	System type	Scoring range	Best embryo	Cell number	Frag	Symm	Compaction/ expansion	Cytoplasm features	Cleavage rate	Multi nucleation
Cummins et al. [4]	Embryo quality score (EQS)/embryo development rating (EDR)	2 or 3	Score	1–4	Score 4		+	+		+	+	
Puissant et al. [106]	Embryo scoring	2	Grade	1–4	Grade 4	+	+	+				
Veeck (1991)	Morphological grading	3	Grade	1–4	Grade 1	+	+	+				
Steer et al. [107]	Cumulative embryo score (CES)	3	Grade	1–4	Grade 4	+	+	+				
Giorgetti et al. [108]	4-Point embryo score	2	Point	1–4	4 point	+	+	+				
Ziebe et al. [109]	Embryo quality score	2	Symm or frag score with cell	Symm: 1.0–2.0 Frag: 2.1, 2.2, 3.0, 4.0	≥4 cell +2.1	+	+	+			+	
Van Royen et al. [3]	Top-quality embryo	2 and 3	Score	Frag: A, B, C	Top quality	+	+					+
Desai et al. [110]	Embryo quality store (D3EQ)	3	Score	1–10	Score 10	+	+	+	+/+	Pitting vacuole		

From Ceyhan et al. [88], with permission
+ indicates characteristic included in assessment system; *Frag* fragmentations; *Symm* symmetry

Day 2 after insemination/injection seems to be the most rewarding and practical for nuclear observations. Multinucleation was discovered in 27.4% of the embryos on day 2 vs. only 15.1% on day 3 in a study by Van Royen et al. [112]. This may be partly due to the larger dimensions of day 2 cells and also their better optical accessibility (less overlap) due to the smaller number of cells [112]. Also, Staessen and Van Steireghem [114] reported that 30% of embryos with multinucleation in the 2-cell stage did not show multinucleation in the 3 to 8-cell stage.

Multinucleation indicates a breakdown of one or more cellular events. Multinucleation occurring during the first mitotic event is most likely due to chromosome segregation error and/or mitotic error in the first cleavage [22]. Supporting this contention is the study by Kligman et al. [115] in which more than 70% of embryos displaying multinucleation had aneuploidy when analyzed by FISH. Many studies have correlated the occurrence of multinucleation with an increased rate of aneuploidy and chromosomal abnormalities [115–118]. Multinucleation occurring in embryos while proceeding through the second and third mitotic event may be due to presence of karyokinesis without cytokinesis, accounting for about 30% of multinucleated blastomeres or due to errors in chromosome segregation or other mitotic errors [117].

Various studies have documented the occurrence of reduced in vitro development [104, 119, 120] and lower implantation rate [104, 120–123] when multinucleation is present on day 2. Coupled with day-3 morphology, multinucleation screening on day 2 has enabled the use of single-embryo transfers without any decrease in pregnancy rates compared to the use of more than one embryo and a single selection criterion [121, 123]. Van Royen et al. [112] found that the incidence of multinucleation was positively correlated with factors such as shorter than average stimulations, higher than average number of oocytes retrieved, and higher than average FSH dose for stimulation, and hence, they hypothesized that multinucleation is due to the developmental failure of the oocyte. Also, they found that there is a significant relationship between multinucleation and other negative morphological characteristics of early-cleaving embryos like fragmentation and cleavage rate, and the lowest incidence of multinucleation coincides with minimal fragmentation and optimal cleavage rate [112].

Blastomere Morphology

In human embryos, it has been proposed that the blastomeres in the 4-cell stage differ from each other: The second polar body which remains attached to the embryo until the blastocyst stage is adherent to only one cell, the progeny of which ends up on the outside of the blastocyst at the junction between the inner cell mass (ICM) and the trophectoderm and determines the embryonic axis [22]. Only one cell is capable of forming hCG at the blastocyst stage. Thus, both the tetrahedron shape of the 4-cell embryo and the need for all the cells to be intact are necessary aspects of normal embryonic development [22]. Screening for abnormal day-2 embryos, i.e., 4-cell embryos without the correct orientation, size of blastomeres, or number, is potentially simple and may eliminate embryos that have no potential for later development.

Cell Number

More than 25 years ago, Edwards et al. [124] performed intermittent evaluations of the growing human embryos to determine if they progress through preimplantation development along a predictable timeline, with 95% of them estimated to reach the 2-cell stage by 33.2 h, the 4-cell stage by 49.0 h, the 8-cell stage by 64.8 h, and the 16-cell stage 80.7 h after insemination. Data from single [108] and homogeneous double and triple embryo transfers [109] have confirmed that on day 2, 4-cell embryos, even when showing minor fragmentation, should be preferred for transfer to good-quality 2-cell embryos. Many studies have reported the existence of optimal cleavage rates, with those embryos cleaving either too quickly or too slowly being associated with compromised development [108, 109, 119]. Scott et al. [91] retrospectively considered the impact of day 2 morphometrics on developmental potential and found that when the cell number is considered at 42–44 h post-hCG, even (two and four cells) rather than uneven (three, five, and greater) cell numbers were significantly associated with positive outcome. The 2-cell embryo proceeds through an intermediate 3-cell stage before rapidly cleaving again to form a 4-cell tetrahedron embryo [125–127]. An embryo, which is an even-sized 3-cell, or a 3-cell that is not progressing fast to a 4-cell, is most likely abnormal or has cytokinetic delays [91]. In the study by Scott et al. [91], no embryo scored as 3-cell on day 2 resulted in a successful delivery. Guerif et al. [111] also found that the blastocyst formation rate was higher for 4-cell embryos on day 2 compared to faster cleaving (5–8 cells) or slower cleaving (2–3 cells) embryos. Other studies have also seconded the contention that the presence of greater than 4 cells on day 2 has a negative impact on blastocyst formation [128–130]. Two hypotheses have been suggested to explain this finding [111]: (i) Some blastomeres in these embryos might be rather large anucleate fragments [109], thus explaining the overestimation of their numbers, and (ii) accelerated division might be an indicator of developmental instabilities that would affect the embryo's ability to develop to a blastocyst. Fast developing embryos have been reported to exhibit higher levels of aneuploidy compared to synchronous embryos [131].

Cell Size and Symmetry

Blastomere size and symmetry are relevant parameters that are often underestimated. In embryos with an even number of blastomeres (i.e., 4, 6, or 8 cells) that are, however, asymmetric, the asymmetry likely arises from an uneven distribution of proteins, mRNA, and various organelles, including mitochondria, between the two sister cells. In embryos having an uneven number of blastomeres (i.e., 5 or 7 cells), the asymmetry is more likely to reflect an asynchrony of cell division than an uneven distribution of cytoplasm [88]. It must be remembered though that in normal human development, there is a degree of cleavage asynchrony [22], resulting in 3-, 5-, and 7-cell embryos. This is a normal phenomenon and allows the correct spatial arrangement of the cells in the embryo as it divides [125]. Thus, at any one point, not all cells would be of the same size. However, in an embryo, there can be only three sizes from the 3 to 8-cell stage, and any deviation from this rule will mean that the embryo is fragmented or that one blastomere has arrested [22].

Though there is a need for more studies on the impact of cell asymmetry on embryo development, a few have shown that asymmetry is associated with poor development potential of embryos [104, 123, 132], a markedly reduced implantation rate [104, 108], and a higher incidence of aneuploidy [108]. In the study by Scott et al. [91], no deliveries were recorded when transferred embryos presented with unequal cell size on day 2, regardless of day 1, 3, or 5 morphology. In another group of patients in the same study, only 12 of 132 clinical pregnancies (9%) were from the transfer of at least one embryo with uneven cell size on day 2. Hence, Scott et al. [91] have stated that cell symmetry is a highly significant scoring parameter that has biological significance and should be incorporated into any gated/sequential embryo selection system.

Blastomere cell size has been linked to fragmentation [132] where the degree of fragmentation was correlated with the extent of unevenness of blastomeres. However, the study by Scott et al. [91] did not second this finding, and uneven cell size on day 2 in their study appeared to be more inherent to the embryo and related to cleavage rather than to fragmentation.

It remains to be definitively determined whether spatial arrangement of the blastomeres bears any relationship to developmental competency of the embryos though one study failed to show any significance [133].

Fragmentation

Fragmentation in the embryo refers to the presence of extracellular cytoplasmic fragments not associated with the blastomeres per se [88]. These fragments need to be differentiated from normal *blebs* that occur transiently during cell division [92]. The etiology of fragmentation appears to be linked to abnormalities in the link between nuclear and cytoplasmic cell division [88] that may be associated with apoptosis [134, 135] or anomalies in chromosomal segregation [88]. Antczak and Van Blerkom [92] have questioned the contention that a direct association between fragmentation and apoptosis exists but speculated that fragments per se may provide a trigger for apoptosis if a certain level of developmentally important proteins is eliminated from their polarized domains. The distorted link between nuclear and cytoplasmic cell division may arise from intrinsic problems within the embryo, and/or from developmental abnormalities caused by poor culture conditions [88].

Scoring Systems for Fragmentation

Fragmentation has many phenotypes described which differ in the sizes of the fragments described, the percentage of the volume of the embryo occupied by fragments, and the distribution of this anomaly among the blastomeres [136]. The simple scoring system for fragmentation is based on the volume of the embryo occupied by fragments (1, score 0 = 0%, score 1 = <10%, score 2 = 10–25%, score 3 = >25%) [137]. Alikani et al. [136] proposed a more detailed classification system that took into account the size and location of the fragments relative to the size and position of the nucleated cell (see Table 43.4) [88, 136].

Fragmentation in the embryo is significant as it has been observed that the rate of blastocyst formation decreases significantly with fragmentation on day 2 [111, 119, 138].

Several hypotheses have been suggested to explain the detrimental effects of fragments on embryo development [111]. Van Blerkom et al. [139] suggested that fragments might physically impede cell–cell interactions, interfering with compaction, cavitation, and blastocyst formation. In addition, ultrastructural observations of degeneration in blastomeres adjacent to fragments [140] suggest that fragments might release toxic substances and therefore damage nearby cells [136]. Alternatively, fragments might also reduce the volume of cytoplasm and deplete the embryos of essential organelles or polarized domains [92]. Heavily fragmented embryos are known to present a higher rate of chromosome abnormalities, in particular mosaicism [116, 141, 142]. In the case of moderate fragmentation, it has been suggested that more than the occurrence of fragments per se, the different temporal or spatial patterns of fragmentation have a more profound effect on embryo development [50, 92, 139]. On the other hand, certain phenotypes of minor fragmentation may disappear during in vitro culture, either by lysis or by resorption [104, 139], thereby providing for a high-quality, stage-appropriate embryo on the day of transfer. Interestingly, Alikani et al. [136] showed that microsurgical removal of

Table 43.4 Classification of fragmentation

Pattern	Description	Degree	% Of fragmentation
Type 1	Minimal in volume, and fragments are associated with one blastomere	W1	0–5
Type 2	Localized fragments predominantly occupying the perivitelline space	W2	6–15
Type 3	Small, scattered fragments may be in the cleavage cavity or peripherally positioned	W3	16–25
Type 4	Large fragments distributed throughout the embryonic mass and are associated with asymmetric cells	W4	26–35
Type 5	Fragments appear neurotic, with characteristic granularity and cytoplasmic contraction within the intact blastomeres	W5	>35

From Ceyhan et al. [88], with permission

small fragments can improve implantation potential of embryos by restoring spatial relationship of cells within the embryo and prevention of secondary degeneration, thus mimicking normal embryonic development in the presence of minor fragmentation. This approach to morphology improvement is controversial.

Day 3 Scoring

There have been many different day 3 scoring systems for selecting cleavage stage embryos, all of which have shown a correlation with implantation [107, 110, 143, 144]. As on day 2, cell number (representing cleavage rate), blastomere morphology and fragmentation on day 3 have been correlated with embryo developmental potential. 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 [106, 107], rate of blastocyst formation [145], and pregnancy rates [145, 146] when compared to embryos with less than eight cells. Alikani et al. [119] too found 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. Racowsky et al. [137] demonstrated that those embryos with exactly eight cells on day 3 had the highest implantation rates. They also found that embryos with more than eight cells had a significantly lower implantation rate than those with eight cells (18.1 vs. 24.9%), which may be related to an increased incidence of aneuploidy [147]. The extent of fragmentation on day 3 has been found to be closely related to both implantation following day 3 transfer [137] and also the likelihood of progressing to blastocyst formation and subsequent implantation [88]. Perinatal outcome of such pregnancies with greater than 50% fragmentation has been found to be poor [148].

Van Royen et al. [3, 123] defined a "top-quality embryo" for transfer on day 3 using the following strict criteria: combining day 2 and 3 morphology, four or five blastomeres on day 2 and at least seven blastomeres on day 3 after fertilization, absence of multinucleated blastomeres, and <20% of fragments on day 2 and day 3 after fertilization. In a prospective randomized trial using these criteria, it was found that implantation rate of 42.3% and an ongoing pregnancy rate of 38.5% were obtained for single top-quality embryo transfers as compared to 48.1 and 74%, respectively, for double top-quality embryo transfers [121]. However, the patients in the study were heavily selected: Less than 25% of patients during the time of the study had embryos of sufficient quality to have a single-embryo transfer [6]. The same group [10] conducted a retrospective analysis of single-embryo transfers using the strict top-quality criteria, and they found that the average number of embryos transferred decreased from 2.265 (in 1998) to 1.79 (in 2001), and the multiple pregnancy and twinning rates dropped from 33.6 and 29.5% (in 1998) to 18.6 and 16.3% (in 2001), respectively, while still maintaining the average ongoing pregnancy rate (33.5% per transfer).

Day 4 Scoring

Compaction and morula formation, phenomena which are best seen on day 4 of embryonic development, have been found to be useful indicators of developmental competence [149, 150]. Though compaction begins as early as the 8–16-cell stage on day 3, it is typically observed as a prelude to morula formation on day 4 [88]. The degree of compaction on day 4 has been found to be associated with implantation potential [149], and hence, it is plausible that early compaction on day 3 may also be an important positive predictor of embryonic development [88]. The day 3 score created by Desai et al. [110] included compaction as one of the parameters. Though the association of compaction with pregnancy rate was not found to be significant in the study by Desai et al. [110], Skiadas et al. [150] found that early compaction was significantly associated with implantation depending upon the degree of fragmentation: In optimal embryos on day 3 (≥8 cells and displaying <10% fragmentation), early compaction was associated with a significantly higher implantation rate as compared to embryos with ≥ fragmentation [150].

There are only two studies that have studied the impact of embryo morphology parameters on day 4 such as compaction, degree of fragmentation, and cytoplasmic vacuolization on further embryonic development [150], and both suggest

that these parameters could be useful markers. A potentially informative scoring system for day 4 of embryonic development is the one suggested by Feil et al. [151]: grade 1, early blastocyst, with cavitation or compacted embryo; grade 2, grade 1 compacted morula with one or more morphological anomaly; grade 3, partially compacted embryo with vacuoles or excessive fragmentation present or embryo with eight cells or more and without any signs of compaction; and grade 4, embryos with eight cells or more, with no signs of compaction and having vacuoles or excess fragments, or embryos with less than eight cells and with no sign of compaction. In their study [151], day 4 and 5 single-embryo transfers resulted in similar ongoing pregnancy rates of 38.7 and 32.1%, respectively. More studies are needed before this day 4 grading system can be accepted as a viable option to the accepted day 5/6 scoring system of Gardner et al. [152]. Also, though D4 evaluation may be potentially valuable, generally leaving embryos undisturbed in culture on day 4 is a more standard approach, relying primarily on earlier parameters or blastocyst development.

Day 5 or 6 Scoring of the Blastocyst

The assessment of the embryo at either the pronucleate or cleavage stage can be at best considered an assessment of the oocyte, and thus, it provides limited information regarding true embryonic developmental potential. There is a strong paternal effect on development that is mainly evident after the 8-cell stage [153, 154]. Seli et al. [155] have established that a significant negative correlation exists between DNA damage in ejaculated spermatozoa and subsequent blastocyst development.

There is evidence that embryo selection on day 2 or 3 based on morphological criteria may be imprecise, resulting in transfer of embryos that are abnormal or arrest at later developmental stages [156–158]. Consequently, extended culture till blastocyst stage and day 5 or 6 scoring of the blastocyst for the selection of the best embryo may be beneficial.

The blastocyst is very dynamic and since it grows rapidly, ideally, it should be assessed and scored sequentially. At approximately 154 h after hCG or 112–114 h after insemination, the blastocyst should have a defined blastocoel, a distinct ICM protruding into the blastocoel cavity, and a ring of evenly spaced and sized trophectoderm cells [22]. Any discrepancy in this sequence of events could lead to developmental problems.

If compaction, which normally begins at the 8–16-cell stage, starts too early, it can lead to the formation of a trophoblastic vesicle, where all the cells are allocated on the outside, leaving none for the ICM [159]. As compaction progresses, refractile bodies in the cells begin to fuse in defined areas to form the blastocoel. If these vesicles form too early, there is the appearance of a paving-stone formation which has a negative impact on blastocyst formation and implantation [22]. If the blastocysts are very expanded by this stage, they may have lowered viability [160]. Also, blastocysts that have finger-like projections across the blastocoel which do not break down with time should be avoided [22].

ICM dimensions have also been found to be highly indicative of blastocyst implantation potential [161]. Richter et al. [161] found that day 5 expanded blastocysts with ICMs of >4,500 μm^2 implanted at a higher rate than those with smaller ICMs (55 vs. 31%). Day 5 expanded blastocysts with slightly oval ICMs implanted at a higher rate (58%) compared with those with either rounder ICMs (7%) or more elongated ICMs (33%). Implantation rates were highest (71%) for embryos with both optimal ICM size and shape [161].

A number of scoring systems for blastocysts have been proposed that take into account some of the above concerns and show correlations with implantation [128, 152, 162]. However, the score devised by Gardner et al. [152] is most commonly used and will be described here. It was determined in the mouse model that total cell number, ICM cell number, and glycolysis had the strongest correlation with embryo viability [6]. Blastocyst formation and hatching, however, were poorly correlated with pregnancy outcome [6]. An alphanumeric scoring system was therefore developed that takes into account three aspects of blastocyst morphology: degree of expansion, ICM development, and trophectoderm development (Fig. 43.1) [163]. In a retrospective analysis of the blastocyst score and subsequent implantation rates, it was determined that when a patient received two top-scoring blastocysts, i.e., 3AA or higher, an implantation rate of 70% was attained [152]. Conversely, when a patient received slow or low-scoring blastocysts, i.e., <3AA, the implantation rate fell to 28% [152].

Interestingly, Richter et al. [161] found that blastocyst diameter and trophectoderm cell numbers were unrelated to implantation rates. In their study, pregnancy rates were higher for day 5 transfers of optimally shaped ICMs compared with day 5 transfers of optimally sized ICMs. They have suggested that this lack of association between trophectoderm cell numbers and implantation rates casts doubts on the appropriateness of inclusion of the trophectoderm grade in the scoring system defined by Gardner et al. [152]. However, this lack of association is surprising, given that it is the trophectoderm that forms the initial connection to the uterine wall and develops into the placenta and associated tissues supporting embryonic development [161]. It may be that the sample sizes used in the study were insufficient to detect existing relationships. It is also possible that some as yet undetermined characteristic of the trophectoderm layer could be indicative of blastocyst viability and implantation potential [161].

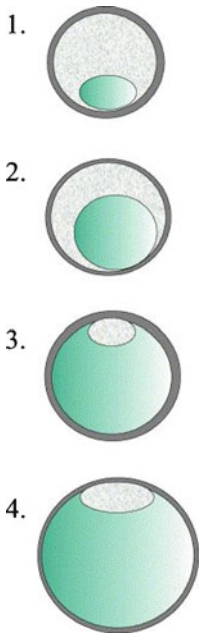

Fig. 43.1 Blastocyst scoring system used to select embryos for transfer. Initially, blastocysts are given a numerical score from 1 to 6 based upon their degree of expansion and hatching status: (1) early blastocyst, the blastocoels being less than half the volume of the embryo; (2) blastocyst, the blastocoels being greater than or equal to half of the volume of the embryo; (3) full blastocyst, the blastocoel completely fills the embryo; (4) expanded blastocyst, the blastocoel volume is now larger than that of the early embryo and the zona is thinning; (5) hatching blastocyst, the trophectoderm has started to herniated though the zona; and (6) hatched blastocyst, the blastocyst has completely escaped from the zona. The initial phase of the assessment can be performed on a stereo microscope. The second step in scoring the blastocysts should be performed on an inverted microscope. For blastocysts graded as 3–6 (i.e., full blastocysts onwards), the development of the inner cell mass (ICM) and trophectoderm can then be assessed; ICM grading: (A) tightly packed, many cells; (B) loosely grouped, several cells; (C) very few cells. Trophectoderm grading: (A) many cells forming a tightly knit epithelium, (B) few cells, and (C) very few cells forming a loose epithelium (from Gardner and Schoolcraft [163], with permission)

Table 43.5 Graduated embryo score of cleavage stage embryos

Evaluation	Hours after insemination	Developmental milestone	Score
1	16–18	Nucleoli aligned along pronuclear axis	20
2	25–27	Cleavage regular and symmetrical	30
		Fragmentation[a]	30
		Absent	25
		<20%	0
		>20%	
3	64–67	Cell number and grade[b] 7CI, 8CI, 8CII, 9CI	20
Total score			100

From Neuber et al. [177], with permission

[a]If the embryo was not cleaved at 25–27 h, grading of fragmentation should occur at the 64- to 67-h evaluation if the embryo reached the 7-cell stage and had <20% fragmentation

[b]Grade I = symmetrical blastomeres and absent fragmentation, grade II = slightly uneven blastomeres and <20% fragmentation, grade III = uneven blastomeres and >20% fragmentation. Grade A embryos are seven or more cells with <20% fragmentation

Sequential Multiday Scoring Systems

A concept that includes many of the above morphological factors is one that attempts to have a continuous scoring system whereby multiple multiday parameters are used to select the best embryo as opposed to a single evaluation performed shortly before transfer. The possibility of combining or integrating findings from observations at different periods of time to improve the assessment of embryo viability has been explored in many studies [111], and several numerical scoring systems have been proposed. Racowsky et al. [164] have classified these systems as follows: morphological observations and the allotment of scores [3, 108, 110, 165, 166], the application of logistic regression analysis [105, 111, 167–169], class probability tree analysis [170], a case-based reasoning system [171], decision tree data mining [172], and automated pattern analysis [173].

Fisch et al. [174] proposed the graduated embryo score (GES) to evaluate parameters from the first 3 days of development (pronuclear morphology, early cleavage, and day 3 morphology) following a study using 1,245 embryos (see Table 43.5) [165, 174]. Using the GES score, they found that embryos scoring 90–100 had 64% blastocyst formation compared with 31% scoring 70–85 and with 11% scoring 30–65 [174]. In patients with at least one transferred embryo scoring ≥70, the pregnancy rate was 59% compared with 34% if all embryos scored <70. Among embryos scoring 70–100, an implantation rate of 39% was seen, compared with 24% among embryos scoring 0–65. In later studies, they further validated their score and found prospectively that transfer of one or more embryos with GES ≥ 70 predicts pregnancy and implantation rates better than a single morphological evaluation on day 3 [165]. Also, they found that day 3 transfers using GES and sHLA-G improve predictive accuracy of ART outcome, allowing single-embryo transfers, with age ≤37 years being an important qualifier [175].

Neuber et al. [176] examined morphological parameters of 1,550 individually cultured embryos, on all 5 days of development, and proposed a multistep sequential embryo assessment scoring system. Figure 43.2 shows a number of scenarios for sequential assessment predicting blastocyst development [176]. They found a significant positive relationship between early-cleaving 2-cell embryos and subsequent good-quality ≥4-cell, ≥7-cell, and blastocyst development. Combining all parameters, they found that a developing embryo showing PN symmetry with early cleavage, and subsequent good ≥4-cell and ≥7-cell cleavage, has a one in two chance of developing into a good-quality blastocyst [176]. Neuber et al. [177] later

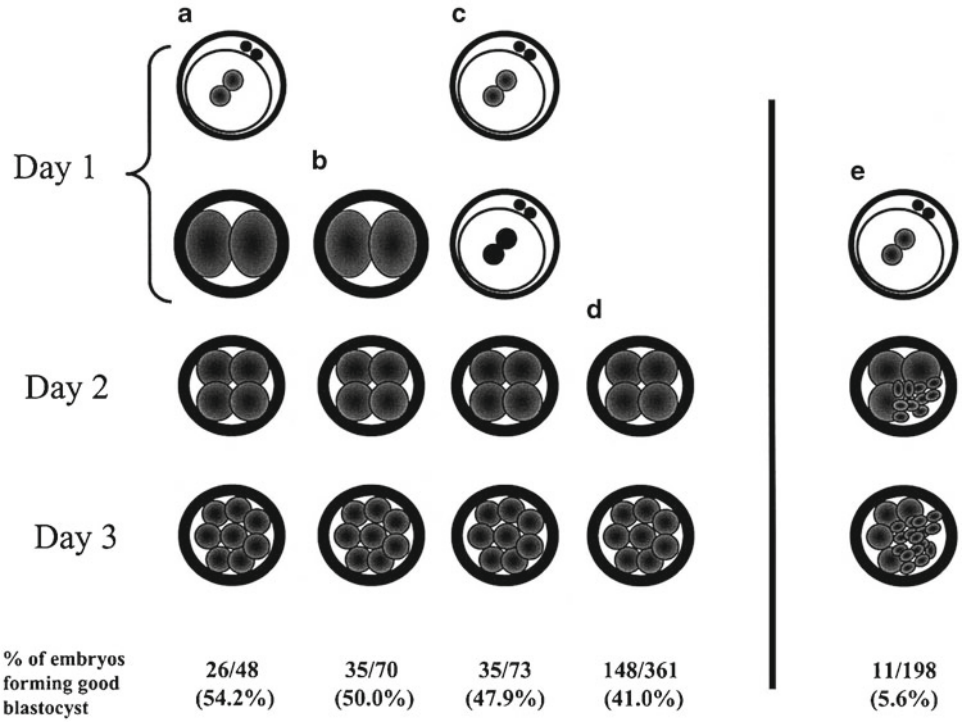

Fig. 43.2 Sequential assessment of individual embryos and their ability to reach the blastocyst stage on day 5. Percentage development to the blastocyst stage is calculated from the number of blastocysts divided by the number of embryos showing the sequential cleavage characteristics. (**a**) All embryos showing PN alignment, early cleavage, and good-quality >4- and >7-cell development. (**b**) All embryos showing early cleavage and good-quality >4- and >7-cell development. (**c**) All embryos showing PN alignment, PN breakdown, and good-quality >4- and >7-cell development. (**d**) All embryos showing good-quality >4- and >7-cell development only. (**e**) All embryos showing intact PN and poor quality >4- and >7-cell development (from Neuber et al. [177], with permission)

developed a computer algorithm based on the sequential assessment tool which took into account quantifiable visual characteristics of multiple stages of development, beginning with pronuclear alignment, the length of the first cell cycle, and cleavage stages (cell number and morphological appearance) up to day 3 of insemination.

Rienzi et al. [178] used a combined pronuclear, cleavage-stage, and day 3 scoring system to state that in a selected population of good prognosis patients, implantation potential of day 3 and day 5 embryos is equal. Using a multiple-step scoring system, Rienzi [179] later reported 77% blastocyst formation on day 5 for embryos with normal pronuclear stage and early cleavage on day 1; 4–5 cells with equal blastomere size, <10% fragmentation and no multinucleation on day 2; and >6 cells with equal blastomere size, <10% fragmentation, and no multinucleation on day 3. However, the proposed values that defined these scores were attributed quite arbitrarily since they were only based on observed frequencies [111].

Gardner and Sakkas [6] too have proposed a multiday scoring system combining morphological parameters evaluated 18–19 h (pronuclear, cytoplasm, NPB, and polar body morphology), 25–26 h (early cleavage and nuclear membrane breakdown), 42–44 h (multinucleation, cell number, and fragmentation), 66–68 h (multinucleation, cell number, and fragmentation), 94–96 h (compaction, signs of blastocoels formation), and 106–108 h (trophectoderm, ICM, and blastocoel cavity) postinsemination/ICSI. The maximum score for a perfect embryo over 5 days would be 180. This score needs to be prospectively studied for its efficacy.

Key Issues Regarding Sequential Morphological Assessment

Though the idea of sequentially assessing embryos seems promising, conflicting results exist overall, and there is currently no consensus on (1) the optimum day(s) for evaluation, (2) the optimum set of variables that should be used in a predictive model, (3) the statistical methods used for data analyses and interpretation, (4) the scoring system to be used for embryo selection, and (5) the model performance benchmarks for future comparison. Answers to these questions are necessary so that quantitative comparisons can be made about the relative efficacies of the different evaluation protocols [88, 164].

There are several concerns regarding the various morphological grading systems:

1. Most studies evaluating embryo viability using pronuclear stage [65, 66], early-cleaving 2-cell embryos [95, 99, 102], and culture until the blastocyst stage [152] have involved grouped embryos. Since the developmental fate of each embryo was not traceable to a viable implantation and there is no proof that the predicted top-quality embryo was the one that implanted out of all the embryos transferred, the conclusions made must be interpreted with caution [88].
2. Results of the various studies may be contradictory due to the multifactorial situations in which nearly all of these studies were performed, namely, the use of different culture media, stimulation protocols, and differences in timing of fertilization assessments, for example, the inclusion of early cleavage in the scoring system used [64].
3. Early development especially the pronuclear stage is very dynamic, and evaluation of early-stage embryo requires testing to be accomplished within strict time windows. In a busy laboratory, adherence to such strict timelines is difficult. Moreover, evaluations are usually accomplished during regular work hours that may not coincide with the optimal or most predictive times to evaluate an embryo.
4. A *numerical scoring system* for selection is frequently based on assignment of seemingly arbitrarily weighted values [88] without multivariate analyses or Spearman's correlation being performed [133, 178]. And hence, it is plausible that the obtained score does not precisely predict the implantation potential. Also, categorical grouping of continuous variables (e.g., fragmentation) may lead to contradictory results.
5. Most of the studies on which the scoring systems are based are retrospective with very few reporting prospective assessment with demonstrated improvement in selection [102, 165]. Rigorous prospective testing of any specific algorithm is necessary to prove that its application does improve embryo selection [88].
6. Many studies have involved datasets involving both day 3 and day 5 transfers [91, 165], an approach likely to reduce utility of the analyses due to various confounders relating to patient selection bias, culture influences (e.g., probable improved overall quality of embryos cultured to day 5, possible loss of developmentally competent day 3 embryos not supported by extended culture conditions), and variances in uterine receptivity [88].
7. Studies based on multiple embryo transfers may introduce further confounding factors due to the possible interembryo cooperation/interaction whereby a poorer-quality embryo may either increase or decrease the likelihood of a better quality embryo implanting or, conversely, a better quality embryo may enhance the independent implantation potential of one considered of poorer quality [88, 180].
8. Precision and consistency of scoring systems among embryologists (particularly for embryos of marginal quality) within and across laboratories must be taken into consideration [88, 133] as it may reduce the accuracy of the system.
9. The potential benefits of the multiday scoring systems must be weighed against the possible detrimental effects of environmental perturbations (light exposure, temperature, pH shifts, etc.) caused due to increased time taken for conducting the strict evaluations.

Racowsky et al. [164] have called into question the need for a sequential system of assessment. They assert that logically, an embryo that shows normal development on day 3 is likely to have exhibited normal development earlier in culture [88]. They have claimed that unpublished preliminary data from their group, utilizing multivariate analysis of a dataset of single-embryo transfers of embryos cultured individually, suggest that no additional benefit is accrued from early cleavage and day 2 assessments over that obtained exclusively from evaluation on day 3. They found that there is considerable overlap in the morphological appearance of those embryos that successfully implant vs. those that fail. Guerif et al. [111] have also advocated the need to search for additional criteria, including the ability of the blastocyst to develop. They prospectively and sequentially evaluated (pronuclear morphology on day 1; early cleavage, cell number, and fragmentation rate on day 2) in 4,042 individually grown embryos and found that the combination of all four parameters allowed the prediction of blastocyst development with an area under the receiver operating characteristics curve of 0.688, which represents a fairly low prediction of embryo viability.

However, it is plausible that early developmental insults resulting in morphological defects that can be seen on day 0 or 1 may not be visually apparent when the embryo is examined on day 3 or 5 but may manifest later in the form of lowered implantation potential and reduced pregnancy rate. More stringent studies comparing sequential assessment with single assessment are required before any definitive conclusions can be made.

Summary

Selecting embryos for transfer is a large part of the *art* in assisted reproductive technology. [165]. A future in which single-embryo transfers are the norm due to legal or ethical issues is well within sight. Hence, development of a universally accepted accurate, noninvasive, easy, simple, and quick grading system to select the best possible embryo with maximum implantation potential is one of the greatest challenges in in vitro fertilization today. A significant number of human preimplantation embryos undergo deviant development in

vitro, failing to follow the expected normal developmental timeline. Hence, it would appear prudent to incorporate as much information gathered at the pronuclear and cleavage stages into the final assessment as possible. This realization has prompted the hypothesis that multiple evaluations through early preimplantation development may improve selection compared with a single evaluation performed shortly before transfer. Such an evaluation would logically take into account every developmental hurdle in the preimplantation development period of the embryo, thus improving predictive accuracy. Various scoring systems, that include various different combinations of multiday evaluations, have been proposed. Most have been found to be associated with improved developmental competency of the embryo. However, consensus on most aspects of sequential analysis remains elusive, including the parameters used in the different scoring systems, optimal day(s) or time for evaluation, predictive accuracy of the various scores and intergrader consistency of evaluation, and lastly appropriate study design and statistical analysis. There are also issues regarding the reliability of the results due to inability to determine the exact developmental fate of the selected *best embryo* due to grouping of the embryos in most studies. Various metabolic methods of embryo evaluation are now proving to offer precise, objective information about the embryo. However, in the absence of alternatives, the development of a universally accepted sequential scoring system that does a multifactorial analysis of various morphological parameters at each defined hurdle of embryonic development is still an enticing proposition.

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Metabolomics: The *ViaMetrics-E*™ Procedure for Assessing Embryo Viability

44

D. Sakkas, L. Botros, M. Henson, K. Judge, and P. Roos

Abstract

Metabolomics is a new and emerging technology that allows us to measure factors in embryo culture media. The complete array of small-molecule metabolites that are found within a biological system constitutes the metabolome and reflects its functional phenotype. Metabolomics is the systematic study of this dynamic inventory of metabolites, as small molecular biomarkers representing the functional phenotype in a biological system. Using various forms of spectral and analytical approaches, metabolomics attempts to determine metabolites associated with physiologic and pathologic states. Metabolic studies of embryos indicate that embryos that result in pregnancy are different in their metabolomic profile compared to embryos that do not lead to pregnancies. When performing an embryo assessment using these new technologies, two important factors must be kept in mind. First, whatever the embryo assessment technique, the inherent quality of the embryo is responsible for the score or assessment characteristics. Hence, a poor embryo is not improved by these technologies; it is only assessed as having lower reproductive potential. Second, the values obtained by a diagnostic platform are only important for an individual patient. Therefore, it is important to be able to pick the best embryo from an individual patient's cohort; it is this assessment which can potentially improve a couple's chance of achieving a pregnancy per cycle.

Keywords

Metabolomics • *ViaMetrics-E* procedure • Embryo viability testing • Assessment of embryos • Assisted reproduction technology

Assisted Reproduction Technology

Success of ART

The treatment of infertility has been a technological challenge as far back as the fourteenth century when there are accounts of Arab peoples using artificial insemination on horses. Even in the third century AD, records show that Jewish thinkers were discussing the possibility of accidental or unintentional human insemination by artificial means. In 1978, the culmination of knowledge in the technologies associated with IVF led to the first IVF birth in the world [1]. Further improvements in the early 1980s by groups in Australia led to births after drug-induced superovulation in the mother, the world's first frozen embryo baby [2], and the first donor egg baby [3].

Utilization of assisted reproductive technologies (ART) continues to increase annually, with over 140,000 treatment cycles being initiated in the United States alone in 2008. This trend is driven by the steady improvement in ART delivery rates, the improved access to care in many areas, and the

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relative ineffectiveness of other treatment options. At the current time, more than 1% of all children born in the United States and Europe are from ART-related conceptions, and in Denmark, for example, the proportion of ART births out of all births is at 3.9%.

Risks of ART Related to Multiple Embryo Transfers

The ability to induce superovulation in the mother has led to the availability of numerous embryos per treatment cycle. As a consequence, however, the success rates established through IVF are attained, in many cases, only through the simultaneous transfer of multiple embryos. For the <35 age group in 2008 in the United States, an average of 2.2 embryos per patient were transferred leading to a 41.3% delivery rate per initiated IVF cycle and an overall multiple birth rate of 35.2%. The live birth results for older age groups decrease dramatically even though more embryos are transferred. The relative risk for multiple births in the 35–40 age group, however, remains above 25% [data from www.sart.org].

The risks related to multiple gestations are well known and include preterm delivery, low birth weight, and dramatic increase in the relative risk for cerebral palsy [reviewed by [4]]. These complications lead to a higher incidence of medical, perinatal, and neonatal complications and a tenfold increase in healthcare costs compared to a singleton delivery [5]. Decreasing the prevalence of multiple gestations while maintaining or improving overall pregnancy rates remains the most significant contemporary goal of infertility research.

Toward Single Embryo Transfer

In a number of countries, including Norway, Sweden, Denmark, Belgium, and England, the dangers associated with multiple pregnancies have been allayed by legal restrictions on the number of embryos that can be transferred in a single IVF cycle. For example, in most Scandinavian countries and Belgium, the government has set a legal limit of single embryo transfer (SET) (i.e., only one embryo to be transferred per cycle) for specific patient groups, while many other European countries have restricted the number of transferred embryos to a maximum of two. In other parts of the world, where no legal restrictions exist, the onus is on the individual clinic (as well as the patient) to decrease the number of embryos transferred so that an acceptable balance can be achieved between the risks associated with multiple gestations and "acceptable" pregnancy rates. This approach has worked well in countries such as Finland and Australia which enjoy remarkably high rates of SET. Current indications are that in the future clinics in the United States and other countries, currently lacking legislation, will be compelled via legal, financial, and/or moral obligation to restrict the number of embryos transferred in order to minimize the risk of multiple gestations.

Implementing SET in the Laboratory

A major issue in limiting the number of embryos transferred is the apparent inability to accurately estimate the reproductive potential of individual embryos within a cohort of embryos. Great progress has been made using the existing selection techniques, which largely encompasses morphological evaluation, but many limitations exist. Morphological assessment has been a stalwart in the armory of the embryologist for selecting which embryo(s) to replace. Since the early years of IVF, it was noted that embryos cleaving faster and those of better morphological appearance were more likely to lead to a pregnancy [6, 7]. Morphological assessment systems have evolved over the past decade and in addition to the classical parameters of cell number and fragmentation, numerous other characteristics have been examined including pronuclear morphology, early cleavage to the 2-cell stage, top quality embryos on successive days, and various forms of sequential assessment of embryos [see reviews by [8–10]]. In addition, the ability to culture and assess blastocyst-stage embryos has also significantly improved the ability to select embryos on the basis of morphology [11].

Faced with the scenario that the worldwide IVF community, will, in the future, have to select only one or two embryos for transfer, we will be forced to make certain changes. The first may be to rely on less aggressive stimulation protocols, hence generating a lower number of eggs at collection [12, 13]. The second is to improve the selection process for defining the quality of individual embryos so that the ones we choose for transfer are more likely to implant.

Assessing the Embryo for Transfer

Noninvasive Assessment of Embryo Culture Media

Historically, it is accepted that there is a relationship between metabolic parameters and embryo viability. In 1980, Renard et al. [14] observed that day-10 cattle blastocysts which had an elevated glucose uptake developed better, both in culture and in vivo after transfer than those blastocysts with a lower glucose uptake. Subsequently, in 1987, using noninvasive microfluorescence, Gardner and Leese [15] measured glucose uptake by individual day-4 mouse blastocysts prior to

transfer to recipient females. Those embryos that went to term had a significantly higher glucose uptake in culture than those embryos that failed to develop after transfer. Further animal studies by Lane and Gardner [16] showed that glycolytic rate of mouse blastocysts could be used to select embryos for transfer prospectively. Morphologically identical mouse blastocysts with equivalent diameters were identified using metabolic criteria, as "viable" prior to transfer and had a fetal development of 80%. In contrast, those embryos that exhibited an abnormal metabolic profile (compared to in vivo developed controls) developed at a rate of only 6%. Clearly, such data provide dramatic evidence that metabolic function is linked to embryo viability.

Human Embryo Studies Assessing Metabolism

A limited number of studies have been performed on nutrient uptake and the subsequent viability of the human embryo. In a retrospective analysis, Conaghan et al. [17] observed an inverse relationship between pyruvate uptake by 2- to 8-cell embryos and subsequent pregnancy. In a study on human morulae and blastocysts of different degrees of expansion, no conclusive date was generated on the ability of nutrient consumption of utilization to predict pregnancy outcome [18]. Unfortunately, in both the above studies, the medium used to assess embryo metabolism was a simple one and was not reflective of current commercial media which are more complex in nature. In contrast, Van den Bergh et al. [19] showed that in patients who conceived following blastocyst transfer, embryos had an elevated glucose uptake and a higher oxidative rate compared to those blastocysts which failed to establish a pregnancy. Significantly, in the work of Van den Bergh et al. [19], a complete medium was used for the metabolic assessment, thereby alleviating the culture-induced metabolic stress.

Furthermore, two studies have determined the relationship between embryo nutrition and subsequent development in vitro [20, 21]. Gardner et al. [20] determined that glucose consumption on day 4 by human embryos was twice as high in those embryos that went on to form blastocysts. They also found that blastocyst quality affected glucose uptake. Poor quality blastocysts consumed significantly less glucose than top-scoring embryos. In studies on amino acid turnover by human embryos, Houghton et al. [21] determined that alanine release into the surrounding medium on day 2 and day 3 was highest in those embryos that did not form blastocysts. Brison et al. [22] have reported that changes in concentration of amino acids in the spent medium of human zygotes cultured for 24 h in an embryo culture medium containing a mixture of amino acids using high-performance liquid chromotography. They found that aspargine, glycine, and leucine were all significantly associated with clinical pregnancy and live birth.

Other Noninvasive Assessment Techniques

Other techniques have also been reported to measure metabolic parameters in culture media; however, they have yet to be tested in a clinical IVF setting. These include the self-referencing electrophysiological technique, which is a noninvasive measurement of the physiology of individual cells and monitors the movement of ions and molecules between the cell and the surrounding media [23, 24]. An alternative approach measures oxygen consumption of developing embryos using a microsensor system. Interestingly, although this technology has been shown to correlate with bovine blastocyst development, it was less successful in predicting mouse embryo development [25, 26]. The technology has yet to be assessed in a clinical IVF setting however. Recently, more emphasis has been placed on the relationship between reactive oxygen species (ROS) levels in culture media to the outcome of in vitro fertilization cycles. This idea was first introduced by Nasr-Esfahani and Johnson in 1990 as an explanation of abnormal development of mouse embryos in vitro. In the human, a recent study by Bedaiwy et al. [27] has shown that increasing levels of ROS generation in day-3 in vitro embryo culture media may have a detrimental effect on in vitro embryo growth parameters, as well as clinical pregnancy rates in IVF and ICSI cycles.

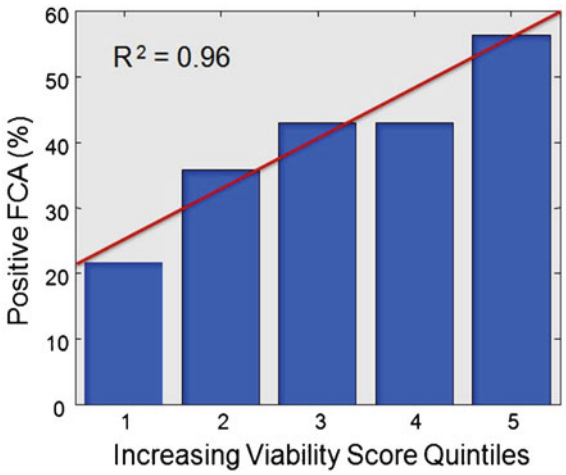
Criteria for Adopting Noninvasive Embryo Assessment in the Laboratory

The inherent ease for the laboratory to assess various morphological markers makes it the preferred assessment technique to transfer embryos. Even with the adoption of more complex forms of assessment, it will still remain as one of the main tools we have in our armory for assessment. For a new technique to be acceptable in a laboratory setting, it must satisfy a number of criteria. It should be able to:

1. Measure a difference in predicting embryo viability without damaging the embryo
2. Measure the change quickly
3. Measure the change consistently and accurately

Metabolomics

The chemical reactions of metabolism are organized into metabolic pathways, in which one chemical is transformed through a series of steps into another chemical, by a sequence of enzymes. A new and emerging technology which allows us to measure factors in embryo culture media is metabolomics. The complete array of small-molecule metabolites that are found within a biological system constitutes the metabolome and reflects its functional phenotype [28]. Metabolomics is the systematic study of this dynamic inventory of metabolites, as



Viability Score Quintile	FCA Positive Rate (%)
1	21.4
2	35.7
3	42.9
4	42.9
5	56.3

Fig. 44.1 Prediction of positive fetal cardiac activity (FCA) in relation to increasing Viability Scores™ when blindly assessing day-5 SET media samples using a preestablished day-5 algorithm. The data analysis includes 72 SET samples from four different clinics using four different embryo culture media. Columns 1–5 contain 14, 14, 14, 15, and 15 SETs, respectively, in each column and represent the SETs with the lowest viability scores in column 1 and increasing Viability Scores™ in columns 2 through to 5, which contains the 15 highest Viability Scores™ (Molecular Biometrics Inc., unpublished data)

small molecular biomarkers representing the functional phenotype in a biological system. Using various forms of spectral and analytical approaches, metabolomics attempts to determine metabolites associated with physiologic and pathologic states [29]. Metabolic studies of embryos indicate that embryos that result in pregnancy are different in their metabolomic profile compared to embryos that do not lead to pregnancies [30].

Investigation of the metabolome of embryos, as detected in the culture media they grow in using targeted spectroscopic analysis and bioinformatics, may therefore divulge these differences. In a recent study, Seli et al. [30] established that these differences are detectable in the culture media using both Raman and near-infrared (NIR) spectroscopy. In this study, a total of 69 day-3-spent embryo culture media samples from 30 patients with known outcome (0 or 100% sustained implantation rates) were evaluated using Raman and/or NIR spectroscopy. A regression formula was developed to calculate a relative "embryo viability score"—relating to embryo reproductive potential—which correlated implantation outcome to both Raman and NIR spectral metabolomic profiles. Both Raman and NIR spectroscopic analysis of the spent culture media of embryos with proven reproductive potential demonstrated significantly higher viability scores than those that failed to implant. Interestingly, when human embryos of similar morphology are examined using the same NIR spectral profile, their viability scores vary remarkably in relation to morphology, indicating that the metabolome of embryos that look similar differ significantly. This observation is in agreement with the study of Katz-Jaffe et al. [31, 32], who revealed that the proteome of individual human blastocysts of the same grade differed between embryos, again indicating that embryo morphology is not completely linked to its physiology.

The algorithm established was subsequently used to predict the likelihood of pregnancy from blinded embryo culture media samples. When the algorithm developed was used to blindly test a subgroup of 16 day-3 embryo samples collected at a different centers and cultured using a different type of commercial media, viability scores of embryos with proven reproductive potential were significantly higher compared to embryos that failed to implant [33]. A larger analysis of SET cycles has also been undertaken whereby NIR spectral analysis of frozen day-2 and day-3 embryo culture media samples was performed blinded to outcome. Individual metabolic profiles were established from 7 mL of the samples with each measurement taking less than 1 min. Statistical analysis performed on the metabolic profiles established a viability score (as generated above) that was significantly different ($P<0.001$) between the pregnant and nonpregnant patients. A cutoff value for predicting pregnancy was taken at >0.3. When this cutoff was used to examine embryos of excellent and good morphology that underwent SET, a significant difference was found in the establishment of pregnancy [34].

More recent data have used a semiquantitative approach to apply this technology. A series of studies have been undertaken whereby algorithms for predicting outcomes of days 2, 3, and 5 SETs have been developed and tested blindly against samples unrelated to those used to develop the algorithms. The data confirmed that as the Viability Scores™ generated by the algorithms increase the tendency for the assessed embryo to implant and display fetal cardiac activity (FCA) also increases (Fig. 44.1). Subsequent data have also shown that this pattern is independent of morphology as the ability of the Viability Score™ to relate to FCA is maintained within groups of embryos with the same morphology.

Fig. 44.2 The ViaMetrics™ instrumentation. The temperature stabilizer is on the *left*, and instrument is on the *right* (courtesy of Molecular Biometrics, ©2010 Molecular Biometrics, Inc.)

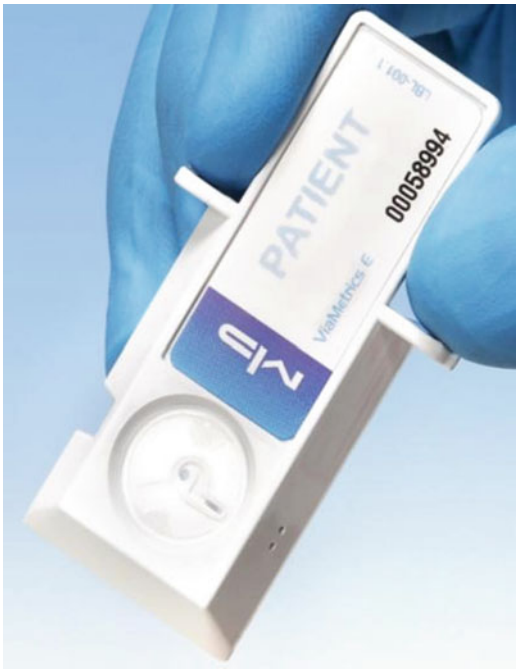


Fig. 44.3 A sample cell that is used to scan the embryo culture media and control media. There are two small injection ports on the side

The *ViaMetrics-E*™ Procedure

ViaMetrics-E™ is a rapid, noninvasive procedure which, when used as an adjunct to morphology, provides an objective measure of embryo viability, thereby helping clinicians and embryologists identify embryos of the highest reproductive potential. Through the use of a highly sensitive method of metabolomic analysis by NIR spectroscopy, the *ViaMetrics-E*™ procedure can be performed on-site in just minutes, requiring only a small amount of spent embryo culture media (i.e., embryo-conditioned media normally discarded after an embryo's culture period).

After an embryo has been removed from a culture media droplet but prior to transfer, a small volume of the spent culture media is sampled and injected into a single-use sample cell. The sample cell is then placed into the *ViaMetrics*™ instrument (Fig. 44.2), which analyzes the sample using NIR biospectroscopy-based metabolomics and proprietary bioinformatics.

The procedure reports a Viability Score™, an objective numerical value which is a quantification of the culture media's metabolomic profile, and which is representative of an embryo's reproductive potential, as determined by its ability to implant and display FCA at 12 weeks. The higher the Viability Score™, the higher the reproductive potential of the embryo. As an adjunct to morphological assessment, the Viability Score™ can be used to rank embryo viability within a single patient's cohort of embryos with similar morphology grades. The entire procedure outlined below will take approximately 15–20 min to scan five embryos.

Embryo Culture

1. Embryos are cultured individually in 20–25 μL droplets. Important note: Any commercially available embryo culture media can be used for the technique.
2. In parallel to the preparation of embryo culture droplets, prepare up to 100 μL of the same media in a separate petri dish or a test tube and place in an incubator for the same period of time as the embryo.
3. The *ViaMetrics-E*™ procedure is able to determine viability when performing days 2, 3, or 5 transfers.

To Prepare Samples for the *ViaMetrics-E*™ Procedure

1. Perform a routine morphological assessment of the embryos on your chosen day of transfer and determine which and how many embryos you will assess.
2. Select the number of embryos, you would like to assess and prepare the sample cells (Fig. 44.3).
3. First, retrieve the total number of sample cells required to scan a single patient's cohort of embryos.
4. Label each sample cell with the appropriate sample identifier (patient ID, embryo number, etc.).
5. Sample cells are then filled with the embryo culture media as follows:
   (a) For each individually cultured embryo in a culture media droplet, collect between 10 and 14 μL of the embryo culture media to be analyzed after the embryo has been moved to another media droplet. All collections are performed with a gel-loading pipette.
   (b) Perform the media collection under a stereomicroscope. Verify that media are collected and that collection of any of the oil overlay is avoided.

(c) Once the sample has been collected, wipe the pipette tip with an unused Kimwipe® to remove any oil residue from the outside of the pipette tip and verify that there are no bubbles in the sample.

6. Insert the gel-loading tip fully into either of the 2-cell injection ports on the side of the sample cell and slowly deposit the embryo culture media sample into the cell channel.
7. Insert the loaded sample cell into the temperature stabilizer (Fig. 44.2) for 4 min before initiating the *ViaMetrics-E*™ procedure.
8. Repeat the above sample cell-loading procedure for each embryo within the patient's cohort.

Scanning a Sample

1. A quality control is performed daily prior to scanning samples by completing three separate scans: a dark cell scan (that blocks the light path), an empty chamber scan, and a reference standard scan.
2. Select the *ViaMetrics-E*™ button on the graphical user interface to initiate the embryo viability assessment procedure (Fig. 44.2).
3. The patient ID and the day of transfer must be entered [culture media type and droplet volume are optional data fields].
4. Retrieve a filled sample cell from the temperature stabilizer. Insert the sample cell into the cell chamber of the instrument (Fig. 44.2) and slide the door shut.
5. Select the Scan button.
6. The sample is first stabilized for 30 s to equilibrate sample temperature before scanning.
7. The sample is then scanned three times to generate three replicate spectra:
   (a) Perform the first replicate scan.
   (b) Slide the sample cell to the top of the cell chamber and then lower it again fully into the chamber.
   (c) Select the Scan Again button. (The scan will be initiated immediately as the temperature is already equilibrated.)
   (d) Perform the second replicate scan.
   (e) Slide the sample cell to the top of the cell chamber and then lower it again fully into the chamber.
   (f) Perform the third replicate, thus completing the embryo culture media scanning process.
8. Repeat steps 5–8 for each embryo sample by pressing the Scan Next Sample button.
9. Once all embryo culture media sample cells have been scanned, select No More Samples and proceed with the Control Scans.

Scanning a Control

1. The Control Scans enable a baseline to be established for each individual embryo's metabolism as the control media never contained an embryo.
2. All embryo culture media samples are now removed from each sample cell by aspiration.
3. Insert a gel-loading tip fully into the sample cell injection port and initiate the aspiration. Alternate from one injection port to the other until the sample cell is empty and dry.
4. Sample cells are then filled with the control culture media as follows:
   (a) For each individual sample cell, collect between 10 and 14 μL of the control culture media.
   (b) Insert the gel-loading tip fully into either of the 2-cell injection ports on the side of the sample cell and slowly deposit the control culture media sample into the cell channel.
   (c) Insert the loaded sample cell into the temperature stabilizer for 4 min before finalizing the *ViaMetrics-E*™ procedure.
5. Repeat the above control procedure for each sample cell within the patient's cohort.
6. Retrieve a filled control sample cell from the temperature stabilizer. Insert the sample cell into the cell chamber and slide the door shut.
7. The instrument will recognize which embryo scan to pair with the control sample cell scan as each individual sample cell is tracked with an electronic tag or radio frequency identification (RFID) tag.
8. Select the Scan button.
9. The control sample is first stabilized for 30 s to equilibrate sample temperature before scanning.
10. The control sample is then scanned three times to generate three replicate spectra:
    (a) Perform the first replicate scan.
    (b) Slide the sample cell to the top of the cell chamber and then lower it again fully into the chamber.
    (c) Select the Scan Again button. (The scan will be initiated immediately as the temperature is already equilibrated.)
    (d) Perform the second replicate scan.
    (e) Slide the sample cell to the top of the cell chamber and then lower it again fully into the chamber.
    (f) Perform the third replicate scan, thus completing the control culture media scanning process.
11. After each control scan is completed, the Viability Score™ will be displayed for each embryo.
12. Repeat steps 7–12 for each control sample cell until the patient's cohort of embryos is complete.
13. Once all control scans are complete, a patient report screen (Fig. 44.4) will appear that provides information about all completed scans. The instrument provides five columns of data:
    (i) Sample number
    (ii) Embryo ID
    (iii) Embryo quality (optional data entered by user)

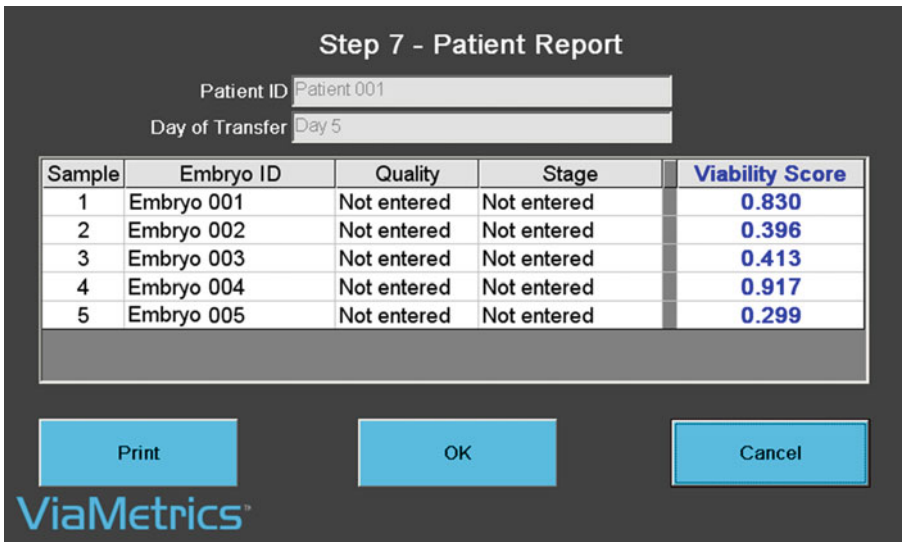


Fig. 44.4 A patient report featuring the patient ID, day of transfer, and the Viability Score™ generated for each individual embryo (Embryo ID) (courtesy of Molecular Biometrics, ©2010 Molecular Biometrics, Inc.)

(iv) Embryo stage (optional data entered by user)
(v) *ViaMetrics-E*™ Viability Score™

Choosing the Embryo for Transfer

1. Once all scans are complete and the Patient Report screen has been provided, the cohort of embryos are ranked according to Viability Score™. The embryo with good morphology and the highest Viability Score™ can be selected for transfer in the case of a SET. In the scenario below, this would be embryo number 004. In the case of a double embryo transfer, embryo numbers 004 and 001 would be selected for transfer.

Conclusion

When performing an embryo assessment using these new technologies, two important factors must be kept in mind. Firstly, whatever the embryo assessment technique the inherent quality of the embryo is responsible for the score or assessment characteristics. Hence, a poor embryo is not improved by these technologies; it is only assessed as having lower reproductive potential. Secondly, the values obtained by a diagnostic platform are only important for an individual patient. Therefore, it is important to be able to pick the best embryo from an individual patient's cohort; it is this assessment which can potentially improve a couple's chance of achieving a pregnancy per cycle. If the scores are overlaid onto the quintile graph of Fig. 44.1, the following scenario could be envisaged for an individual couple (Fig. 44.5). For example in the above scenario where five embryos were assessed from a cohort of equivalent morphological embryos, transferring the highest scoring embryo vs.

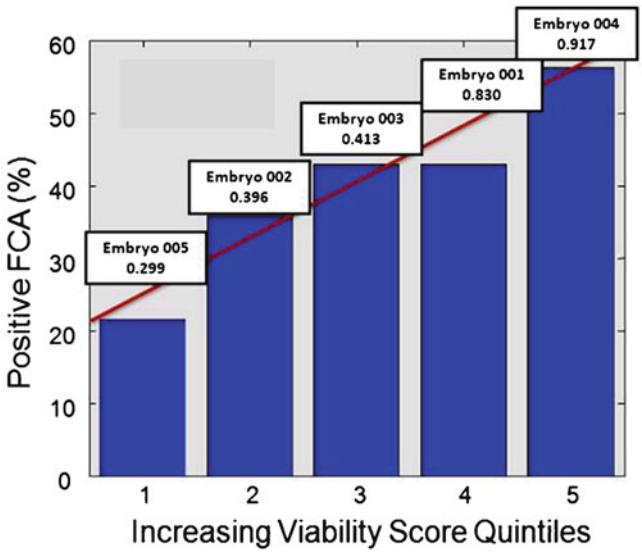


Fig. 44.5 A ranking of the cohort of five embryos displayed in the patient report above in relation to their chance of achieving a positive FCA. Embryo 004 which ranks in the highest quintile of Viability Scores™ has more than double the chance of establishing a positive FCA outcome compared to Embryo 005 which ranks in the lowest quintile

the lowest scoring embryo could potentially improve the couples chance of achieving pregnancy by more than 20%. Therefore, if an assessment technique could help to choose embryos number 001 or 004 instead of number 005, the benefits for a couple's cycle would be significant.

In the near future, the ability to perform a metabolomic assessment, in addition to other forms of assessment, may provide both a benefit in selecting the best single embryo for transfer and also a diagnostic assessment when there are only poor embryos within a cohort.

Addendum Following a limited clinical release their was a voluntary market withdrawal of ViaMetrics-E due to an inability of the instruments to perform measurements consistently and accurately. This parameter is crucial for adopting this type of assessment in the clinical laboratory. It is hoped that an upgraded version of this technology will be released in the future.

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Oxygen Consumption as an Indicator of Oocyte and Embryo Viability

45

Ana S. Lopes

Abstract

Oxygen consumption has been regarded as a valuable parameter for evaluating oocyte and embryo metabolism and seems to provide an alternative approach for assessing their developmental potential prior to transfer. This chapter will detail all aspects related to the oxygen-consumption measurements performed using oxygen microsensors, and describe how oxygen consumption correlates with morphology, developmental stage, sex, chromosomal and genetic background, reactive oxygen species production and viability of the measured oocytes/embryos following transfer. Additionally, it will discuss how oxygen consumption can provide a means to evaluate mammalian embryo quality and possibly complement other selection methodologies currently used within a clinical setting.

Keywords

Oxygen consumption • Embryo metabolism • Oocyte quality • Embryo quality • Oocyte embryo viability • Embryoscope

Oxygen consumption has been regarded as the best indicator of overall metabolic activity [1] and as a valuable parameter for evaluating oocyte and embryo quality [2–6]. Oxygen is essential for the generation of cellular energy (ATP) in the mitochondria, via oxidative phosphorylation. In the oocyte, ATP production depends mostly on the number and activity of mitochondria present in the ooplasm, which increase exponentially with oocyte maturation during follicular growth [7, 8]. Mitochondrial number and function seem to be strongly related to developmental competence, with lower mitochondrial activity and number associated with premature arrest of the oocyte, fertilization failure and reduced embryo development [9–13]. Mitochondrial respiratory activity accounts for 30% of all the oxygen consumed by the embryo at early cleavage stages, a figure that increases to 60–70% at the blastocyst stage [14]. Thus, changes in mitochondrial activity should be directly reflected on the oxygen consumption by the developing embryo. Furthermore, oxygen is a key element of the oviductal and uterine environment, having important roles in regulating embryonic development [15–17]. Therefore, the measurement of oxygen consumption is a promising alternative or complementary approach to the assessment of oocyte and embryo viability.

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Measurements of Oxygen Consumption

History

Several approaches have been used for the measurement of oxygen consumption in oocytes and preimplantation embryos. The first studies used a Cartesian diver technique to measure the oxygen consumption of rabbit ova and mouse embryos [18, 19]. Subsequent studies on human and mice embryos [20, 21] employed spectrophotometric methods to indirectly

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measure oxygen consumption, and Overström et al. [2, 22] employed a multichannel apparatus with oxygen electrodes to assess the oxygen consumption of single mouse and cattle embryos. More recently, the oxygen consumption of mouse and cattle embryos has been measured by ultramicrofluorescence [4, 23], by an automatic scanning electrode [14, 24] and by a scanning electrochemical microscopic technique [25]. However, most of the described techniques are invasive, technically challenging and time consuming or require the use of ultraviolet (UV) illumination, radioactive probes or fluorescent dyes. All of these may have toxic side effects and thus are not suitable for the assessment of embryo viability prior to transfer [26, 27]. The ideal method for measuring oxygen consumption of single embryos should be non-invasive, rapid, simple, accurate, reproducible, and capable of the measurement of oocytes and embryos at different developmental stages, all without interfering with their subsequent viability.

The Use of Oxygen Microsensors

The Nanorespirometer: Single Measurements

The Nanorespirometer (Unisense Fertilitech A/S, Aarhus, Denmark) was developed for measuring the oxygen consumption of individual oocytes/embryos at a specific time or stage of development.

Measurement principle and equipment. The production of linear steady-state oxygen gradients was considered the most fundamental requirement for calculating the oxygen-consumption rates of individual oocytes and embryos [28]. The required equipment has been previously described [27]. The measuring unit is composed of seven fused glass capillaries with a closed glass bottom attached to a Udel® polysulfone disc. It is mounted in a holder and submerged in a beaker containing culture medium, which is maintained in a semi-closed system at 38.5°C (or 37°C, depending on the species) and under a constant flow of humidified air (5% CO_2 in 20% O_2). The beaker containing the culture medium is placed in a large glass incubation chamber filled with distilled water at the right temperature to maintain stable temperature conditions during measurements.

Oxygen measurements. An individual oocyte or embryo is placed into the bottom of each glass capillary while two glass capillaries in the rosette are left empty to serve as reference measurement without respiratory activity (=control). The system is left undisturbed for 1 h until a steady-state linear oxygen gradient is established inside the glass capillaries of the measuring unit. The oxygen concentration gradient generated by the respiration of the embryo (Fig. 45.1) is subsequently determined by measuring the oxygen concentration at consecutive equidistant measurement points in the capillary with an oxygen microsensor (Clark-type oxygen microelectrode).

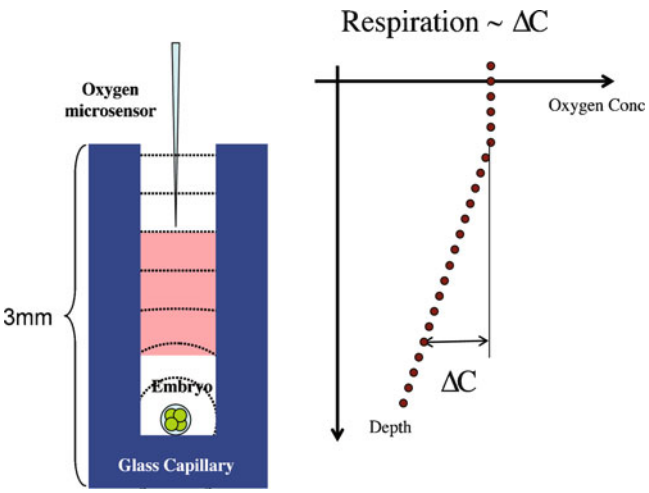

Fig. 45.1 The oxygen-consumption measuring principle. On the *left*, the schematic representation of a glass capillary of the measuring unit (inner diameter 0.68 mm, length 3 mm) with an embryo. On the *right*, an oxygen concentration gradient generated by the oxygen consumption of the embryo and determined by the oxygen microsensor, at consecutive points in the glass capillary. From the slope of the gradient, individual oxygen-consumption rates can be calculated (from Lopes et al. [60], with permission)

The recordings are made by moving the tip of the microsensor down through the media-filled glass capillary, and the oxygen-consumption rates are calculated from the slope of the linear steady-state oxygen concentration gradients measured for each oocyte or embryo (Fig. 45.1) [27].

Evaluation of the nanorespirometer. The Nanorespirometer directly measures the oxygen consumption of an individual intact oocyte or embryo, which distinguishes this approach from those described earlier [4, 24]. Additionally, the Nanorespirometer measures the overall oxygen-consumption rate of an individual embryo lying freely at the bottom of a glass capillary, overcoming the problem of heterogeneity of the blastocyst surface, encountered by Shiku et al. [25] when using the scanning electrochemical microscopy technique. The oxygen gradients resulting from the consumption of oxygen by individual embryos show nearly complete linearity, and first and second oxygen-consumption measurements of the same embryos are highly correlated, demonstrating the consistency of the results. Evaluation of pregnancy rates following transfer of measured embryos [5] was used to assess the effect of employing the Nanorespirometer on subsequent embryo viability. The results showed that pregnancy rates are similar for measured and non-measured embryos.

The EmbryoScope, Version C : Repeated Measurements

The EmbryoScope, Version C (Unisense Fertilitech) allows for repeated and automated measurements of oxygen consumption with simultaneous acquisition of digital images from individual oocytes or embryos during culture for as

long as 7 days. The EmbryoScope, Version C is continuously being developed and consists of a modified version of the Nanorespirometer technology combined with time-lapse videomicrography.

Measurement principle and equipment. The measuring principle used in the EmbryoScope, Version C is similar to that described above for the Nanorespirometer. The equipment has been previously described [5, 6, 29–32]. The EmbryoScope, Version C is built inside a standard in vitro fertilization (IVF) CO_2 incubator (5% CO_2, 19% O_2) and combines a high-resolution microscope system (Leica ×20 LMPLFL objective) with an oxygen microsensor (with a laser positioning system). The microsensor is guided by computer-controlled microcontrollers that guide the alignment of the tip of the microsensor with the wells of the culture dish. The culture dish is an embryo-tested polymer slide with 12 gas-impermeable wells (depth 0.3 mm, diameter 0.7 mm), which is filled with culture medium, overlaid with mineral oil and equilibrated at the right temperature with 5% CO_2 in humidified air for 18 h.

Oxygen-consumption measurements and acquisition of digital images. Oocytes or embryos are loaded into the bottom of each well of the equilibrated culture dish, and the oxygen supply is maintained through molecular diffusion from the overlaying culture medium down through the well. As for the Nanorespirometer, two or three wells of the culture dish should contain only culture medium, in order to serve as reference measurement without respiratory activity (blank). The culture dish is inserted in the incubator 1 h before the start of the measurements so that the oxygen concentration gradient can be established. Calibration of the microsensors is always performed before the measurements as earlier described [29]. Linear oxygen concentration gradients over the oocytes or embryos are repeatedly measured by the oxygen microsensor in all wells of the slide, and calculation of individual oxygen consumption is based on the measured oxygen gradients. The final oxygen-consumption rates of individual oocytes or embryos can be obtained by subtracting the mean value of the blanks in each measurement session from the calculated oxygen-consumption rates.

During embryo development in the EmbryoScope, Version C digital images can be acquired between oxygen-consumption measurements at intervals of 20–120 min. Subsequent analysis of the images allows monitoring the individual embryo development and identifying the time of cell division on embryos that developed beyond the one-cell stage. From the analysis of the time-lapse images, an activity pattern is generated for each individual embryo.

Evaluation of the Embryoscope, Version C. The EmbryoScope™ has been validated using cattle, mouse and human embryos [6, 32–34]. For all three species, the embryos reached the blastocyst stage at rates similar to those normally observed using standard in vitro culture systems. Parallel to the measurements with the EmbryoScope, Version C mouse embryos were measured by *closed respirometry*, and similar mean oxygen-consumption rates were obtained using this approach, which indicates that measurements performed with the EmbryoScope, Version C are accurate and consistent with other systems.

Oxygen Consumption of Oocytes and Embryos

Oxygen Consumption, Morphological Quality and Developmental Stage

The oxygen consumption of human oocytes appears to differ between oocytes and between patients [6, 32, 35]. Oxygen consumption of healthy-looking metaphase II (MII) oocytes on the day of retrieval (day 0) ranges from 0.4 to 0.6 nL/h [6, 20, 32, 35, 36], with fragmented and morphologically abnormal oocytes having lower and higher oxygen consumption, respectively. Correlations between oxygen consumption of human MII oocytes and subsequent fertilization and development in vitro show that higher oxygen consumption is associated with higher rates of correct fertilization (2PN formation), higher implantation rates and a slightly but not statistically significant better embryo quality at 48 and 72 h after fertilization [6, 36]. Nevertheless, the correlation between oxygen consumption, fertilization and development might not be linear, as previously suggested by Leese et al. [37], and this needs to be further investigated. The best developmental potential seems to be associated with MII oocytes with an oxygen consumption of approximately 0.5 nL/h on day of retrieval [36].

During embryo development in vitro, a similar pattern of oxygen consumption has been observed for all species studied (human, cattle and mouse embryos): at the early cleavage stages, oxygen consumption is low and constant, rising gradually at the morula stage and then increasing significantly at the expanded blastocyst stage. A two- to threefold increase between early cleavage stages and expanding blastocyst stage has been reported in the literature [4, 6, 14, 19, 23, 27, 29, 33, 34, 38] (Fig. 45.2). This increase is associated with a higher ATP demand at the time of compaction and blastulation [14, 23, 39, 40], in order to accommodate increased protein synthesis and activity of the plasma membrane Na^+/K^+-dependent ATPase [16, 23], as well as changes in mitochondria morphology and number. Thus, an increase in oxidative phosphorylation at the blastocyst stage is reflected by the oxygen consumed by the developing embryo.

In a study involving cattle embryos, correlations among morphological quality, stage of development and oxygen consumption during the 5 days of culture have revealed four distinct respiratory profiles: (a) an abrupt or gradual drop in oxygen consumption for embryos that did not develop

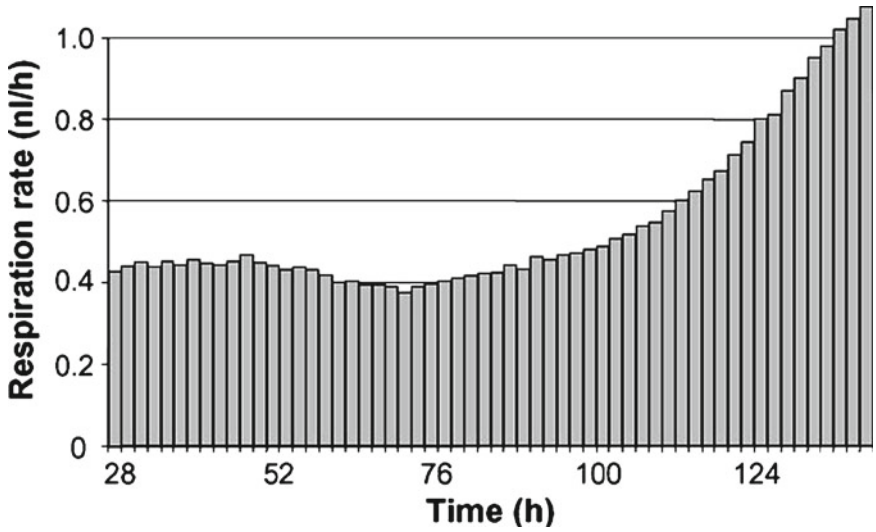

Fig. 45.2 Oxygen-consumption profile of one cattle embryo, measured repeatedly at 2-h intervals from the zygote to the expanding blastocyst stage (from Lopes et al. [60], with permission)

beyond the one-cell or early cleavage stages; (b) stable oxygen consumption (range 0.3–0.4 nL/h), for embryos that do not develop beyond the one-cell or early cleavage stages; (c) a small increase in oxygen consumption for a heterogeneous group of embryos (one-cell embryos, embryos at the early cleavage and poor quality blastocysts) and (d) marked increase in oxygen consumption for high-quality expanding blastocysts, with stable rates at the early cleavage stages (approximately 0.4 nL/h) and a significant increase at the expanding blastocyst (approximately 1.2 nL/h). Two pilot studies with human embryos have revealed similar patterns, with embryos that develop to blastocysts showing oxygen-consumption rates ranging between 1.0 and 1.3 nL/h [32] (Ziebe and Lemmen, unpublished data).

In mouse embryos, higher oxygen-consumption rates at each day or stage were seen in embryos that continued development to the immediately subsequent period [6, 29]. On the contrary, in human embryos a significant decrease in oxygen consumption has been observed from the time of ICSI until transfer on day 3 [29, 32, 41].

An earlier study that assessed the oxygen-consumption rates of individual cattle embryos at short intervals between fertilization and the first division provided evidence of the existence of a major peak of oxygen consumption at the time of fertilization, between 7 and 11 h after sperm penetration. Furthermore, a small respiratory peak was consistently present immediately before or at the exact time of cleavage for every presumptive zygote (Fig. 45.3) [31, 38].

The relationship between oxygen consumption and embryo morphological quality has not been consistent among studies. Some studies suggest that oxygen consumption is higher for embryos judged to be of superior quality, based on morphology [20, 25, 27, 41, 42]. Others showed no correlation between oxygen consumption and morphological evaluation [22, 32], or that blastocysts with a high oxygen consumption have reduced developmental potential [37, 43, 44]. It might be reasonable to assume that the morphologic appearance of an embryo would be linked to its metabolic activity, mainly after major activation of the embryonic genome. This assumption is supported by the fact that oxygen consumption has been correlated with cell number and function [45, 46] and the number of mitochondria [14], which have been shown to be associated with embryo quality. However, the greatest oxygen consumption might not necessarily reflect the most viable embryo. According to Leese et al. [44], less oxygen and nutrients are consumed by a viable embryo, as opposed to a less viable embryo, that needs higher oxygen consumption to repair molecular/cellular damage in developing embryos. Very high oxygen uptake by an embryo can also result directly from damage to the mitochondria, as leakage of the inner membrane results in increased oxygen consumption, but decreased ATP production. Thus, embryos with mitochondria damage have reduced developmental potential regardless of the high oxygen consumption. Considerable variation in oxygen-consumption rates within each morphological category have also been reported [22, 25, 27], further supporting the hypothesis that measurements of the metabolism of individual oocytes or embryos provide additional and valuable information about their subsequent viability and allow for a more accurate evaluation.

Oxygen Consumption, Culture Conditions and Cryopreservation

Oxygen consumption is significantly affected by culture conditions, namely, by glucose [46], serum [47], amino acids and protein content in the culture media [43, 48] and by the oxygen concentration [49]. For instance, the presence of glucose in the culture media has been shown to significantly

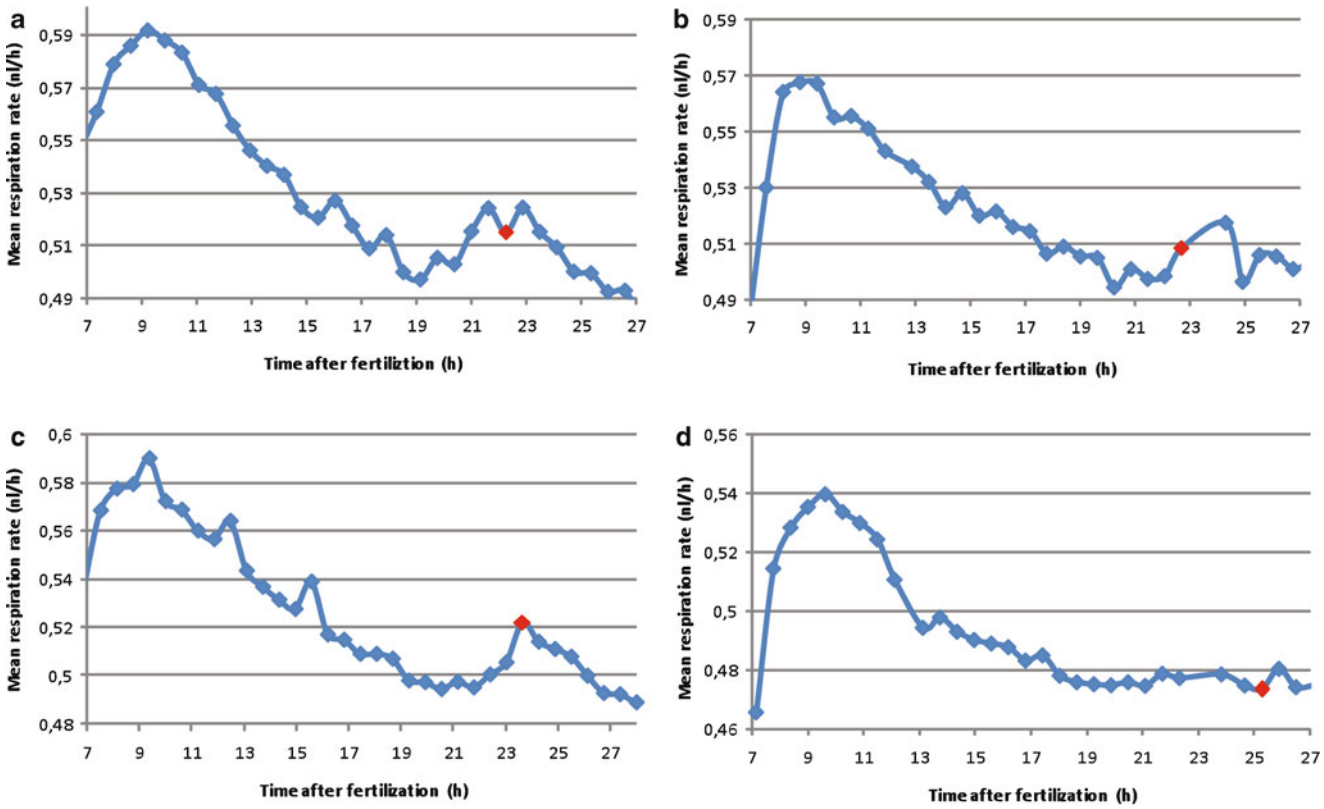

Fig. 45.3 Mean oxygen-consumption rates of cattle embryos that first cleaved at (**a**) 22, (**b**) 23, (**c**) 24 and (**d**) 25 h after IVF. Time-lapse images were acquired during the measurements with the Embryoscope, Version C at intervals of approximately 36 min and used for evaluation of developmental progress. The time of the first cleavage is indicated in each graphic by the *red symbol* (from Lopes et al. [31], with permission)

reduce the oxygen consumption of the embryo [46]. The oxygen consumption of cattle embryos in serum-free media is higher than that in serum-supplemented media, apparently due to a larger proportion of immature mitochondria in embryos cultured under the latter conditions [47]. Alterations in oxygen-consumption rates associated with the addition of exogenous protein to the medium are another example of how culture conditions can interfere with embryonic oxidative metabolism [48].

In vivo- and in vitro-produced embryos develop in distinctly different conditions, and thus, differences at the metabolic level would be expected. For in vivo-produced cattle embryos, oxygen consumption ranged from 0.7 to 0.9 nL/h at the blastocyst stage [5, 23]. At first sight, these rates would seem significantly lower than those observed for in vitro-produced embryos. However, when these two embryo types were measured with the Nanorespirometer, significant differences could not be detected. The discrepancy was attributed to differences in the proportion of embryos within each morphological quality and stage of development. (Day 7 in vivo-produced cattle embryos have fewer cells and are at less advanced stages of development than day 7 in vitro-produced embryos).

As far as cryopreservation is concerned, results are contradictory. Preliminary results suggest that the oxygen consumption of frozen/thawed embryos is comparable to, and follows a pattern that is similar to, that seen during in vitro development of fresh human embryos [32]. Sakagami et al. [50] observed that oxygen consumption was similar before and after vitrification, as long as the vitrification protocol would ensure a high percentage of cell survival. In contrast to these results, Yamanaka et al. [51] showed that the oxygen consumption rate of vitrified blastocysts just after warming was significantly lower than that of non-vitrified blastocysts (blastocysts derived from culture of embryos vitrified at the pronuclear stage).

Oxygen Consumption, Diameter and Sex of the Embryos

The diameter of in vitro-produced blastocysts appears to be related to oxygen consumption, with significantly larger embryos having higher oxygen-consumption rates [2, 25, 27]. Oxygen consumption does not appear to be dependent on the sex of the embryo [27, 30, 42]. Thus, it seems that sexual dimorphism does not occur at the level of oxidative phosphorylation, and oxygen consumption is mostly associated with quality rather than with the sex of the embryos.

Oxygen Consumption and Chromosomal and Genetic Background

The characterization of the expression of key genes involved in embryo metabolism can provide insight on how genetic patterns affect metabolism and thereby embryo viability. In embryos, ATP is produced by glycolysis and by oxidative phosphorylation, processes which require oxygen and glucose uptake, together with the expression and translation of *Glut1* and *G6PD*. One study showed that the expression of *Glut1* and *G6PD* was directly correlated with oxygen consumption [30], which implies that more metabolically active embryos have increased both oxygen and glucose uptake, indicating that oxidative phosphorylation and glycolysis are jointly involved in the maximization of ATP production.

The analyses of aneuploid morulae and blastocysts indicated that alterations in chromosome number were not related to oxygen-consumption rates [32].

Oxygen Consumption and Reactive Oxygen Species Production

Oxidative phosphorylation, essential for ATP production, was an evolutionary breakthrough but came at a cost with the production of reactive oxygen species (ROS). ROS can induce oxidative modifications of the cell components but, when present under physiological concentrations, they participate in normal cell processes as major factors of growth and developmental regulation [52]. Oxygen consumption and ROS were found to be increased at the time of fertilization of cattle embryos, leading to the suggestion that mitochondrial activity is stimulated by Ca^{2+} waves around the time of sperm penetration. A small peak of oxygen uptake was seen to coincide with the first cell cleavage and with increased ROS production [31]. This increase in oxygen consumption is likely associated with a higher energy requirement for the initiation of cell division and a consequence of increased mitochondrial activity. Thus, there are some specific events during embryo development that appear to be associated with a change in oxygen consumption and REDOX state, indicating that both have a role in sperm-mediated oocyte activation and cell cleavage in cattle embryos.

Oxygen Consumption, Age of the Patients and Hormonal Levels

High doses of gonadotrophins, premature LH surges and advanced age of the female patient negatively affect oocyte quality [53–57], which may consequently affect the metabolism of the matured oocyte. This has been confirmed by the lower oxygen consumption measured in human oocytes of patients with high concentrations of baseline FSH [6]. Furthermore, significantly higher oxygen consumption was measured in oocytes of younger patients, which may be related to the higher number and activity of mitochondria in these oocytes. Finally, oxygen consumption of MII oocytes seems to vary according to the type of gonadotrophin used in the stimulation protocol, (high oxygen consumption was observed in oocytes of donors stimulated with FSH, when compared to oocytes of donors stimulated with FSH + hMG or hMG alone) [36], the length of the stimulation protocol (longer stimulations combined with a lower dose of gonadotrophins are associated with higher oxygen consumption by the oocyte) [36] and serum estradiol on the day of hCG administration (higher serum estradiol was associated with less oxygen consumption) [58].

Table 45.1 Pregnancy status according to category of oxygen consumption (high vs. medium vs. low) of bovine in vivo-produced cattle embryos

Oxygen consumption	Pregnant	Non-pregnant
High (>1.10 nL/h)	25% ($n=1$)	75% ($n=3$)
Medium (0.78–1.10 nL/h)	100% ($n=13$)	0% ($n=0$)
Low (<0.78 nL/h)	48% ($n=11$)	52% ($n=12$)

From Lopes et al. [5], with permission

Oxygen Consumption and Viability Following Embryo Transfer

The oxygen consumption of single oocytes or embryos will only become an important evaluation parameter if a direct correlation with subsequent viability can be demonstrated. The correlation between oxygen-consumption rates and subsequent embryo viability, assessed by the pregnancy status of recipients following transfer of pre-measured embryos, has previously been investigated [5, 22, 36, 41, 59]. Overström et al. [22, 59] proposed that embryonic oxygen-consumption rates are positively related to survival rates, but blastocysts were pooled before transfer. Lopes et al. [5] detected a correlation between individual oxygen consumption and pregnancy status of the recipients following single transfer of the pre-measured embryos. Nevertheless, the mean oxygen consumption for embryos producing a pregnancy was only slightly (but not significantly) higher than that observed for embryos which did not produce a pregnancy. Instead, the authors have proposed that an optimal range of oxygen-consumption rates would be associated with a good pregnancy prognosis. It was suggested that three biologically meaningful categories may exist, with a range of oxygen-consumption rates associated with normal development and too low or too high rates related with developmental problems and reduced developmental potential (Table 45.1). Further investigations are currently being carried out using human oocytes and embryos. Preliminary data shows

that higher implantation rates are observed with embryos deriving from oocytes with higher, but not greatest, oxygen-consumption rates [36]. Similarly human embryos that subsequently implanted seem to have a significantly higher oxygen consumption from the time of ICSI until transfer, when compared to embryos that did not implant [41].

Conclusions, Practical Applications and Future Perspectives

Mammalian oocytes and embryos exhibit a high degree of heterogeneity of metabolic activity and developmental capacity. The evaluation of oocyte and embryo quality is controversial, but the measurement of oxygen consumption seems to provide a feasible approach to the assessment of their developmental potential. The EmbryoScope, Version C is a high-resolution device, which provides a non-invasive, fast, accurate and consistent measurement of the oxygen consumption of individual oocytes and embryos throughout their development in culture, without affecting their subsequent viability. These characteristics make the EmbryoScope, Version C a promising tool for assessing oocyte and embryo quality. The combination of oxygen measurements and digital images acquired during embryo development have revealed the general pattern of oxygen consumption from the one-cell stage until the expanded blastocyst stage and have shown common oxygen patterns for embryos arresting during development. Furthermore, the possibility of detection of a respiratory peak at fertilization (signaling sperm-mediated oocyte activation) and at the time of the first cleavage may provide another useful approach to non-invasive evaluation of embryo quality.

The use of oxygen consumption as a diagnostic tool for selection of the most viable embryos has not yet been completely demonstrated. It is still crucial to define and confirm the association between oxygen consumption of individual oocytes and embryos and subsequent fetal development in order to be able to define the optimal oxygen-consumption range.

The oxygen consumption of oocytes and embryos seems to be only partly associated with morphology, suggesting that the embryonic oxygen consumption is a quality indicator on its own. Thus, combining oxygen consumption with morphological evaluation can provide new information on the subsequent viability of oocytes and embryos. The use of oxygen as a parameter of viability may lead to a substantial improvement in the selection of superior oocytes and embryos with higher developmental potential before transfer, which is increasingly required as elective single embryo transfer being progressively implemented worldwide.

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Gene Expression Changes During Human Early Embryo Development: New Applications for Embryo Selection

46

Samir Hamamah, Said Assou, Imène Boumela, and Hervé Dechaud

Abstract

Knowledge of the global pattern of gene expression is important for understanding critical regulatory pathways and key biological process governing human reproduction. Genes involved with intracellular signaling, cell survival or death, pluripotency, transcription and translation, methylation and demethylation, and nuclear reprogramming are the main cellular processes that control the early embryonic development. In this chapter, we relate recent advances on the dynamic genes changes of human early embryo development and the application of DNA microarray as a new emergent technology for embryo selection. Techniques of this type may ultimately lead to noninvasive tests for oocyte or embryo quality, revealing previously hidden information concerning both oocyte and embryonic developmental competence. Increased knowledge of genes and related transcriptional networks operative during early embryonic development should lead to advances in assisted reproductive technologies and preimplantation genetic diagnosis.

Keywords

Early embryo development • Cleavage • Compaction • Blastulation • Gene expression in oocyte maturation • Global gene expression • Embryonic gene expression • Metaphase II oocyte • Apoptosis

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Early embryo development is a complex process that includes a series of important developmental events: final maturation of the oocyte, fertilization, and early preimplantation embryo development. Morphological changes during preimplantation development can be categorized into three main stages: cleavage (increase in cell number), compaction (polarization and cellular flattening), and blastulation (formation of blastocoels). After fertilization, a zygote is formed—the ultimate totipotent cell that can be seen as the most undifferentiated cell type as it gives rise to all cell types and live offspring. Totipotency persists for the very first cell doublings, from the single cell zygote to at least the 4-cell preembryo. The maternal to zygotic transition serves two important purposes: degradation of maternal RNA and protein and activation of the embryonic genome (Fig. 46.1). At morula stage, the preembryo undergoes compaction, with the loss of the cellular distinction between the blastomeres. This is followed by

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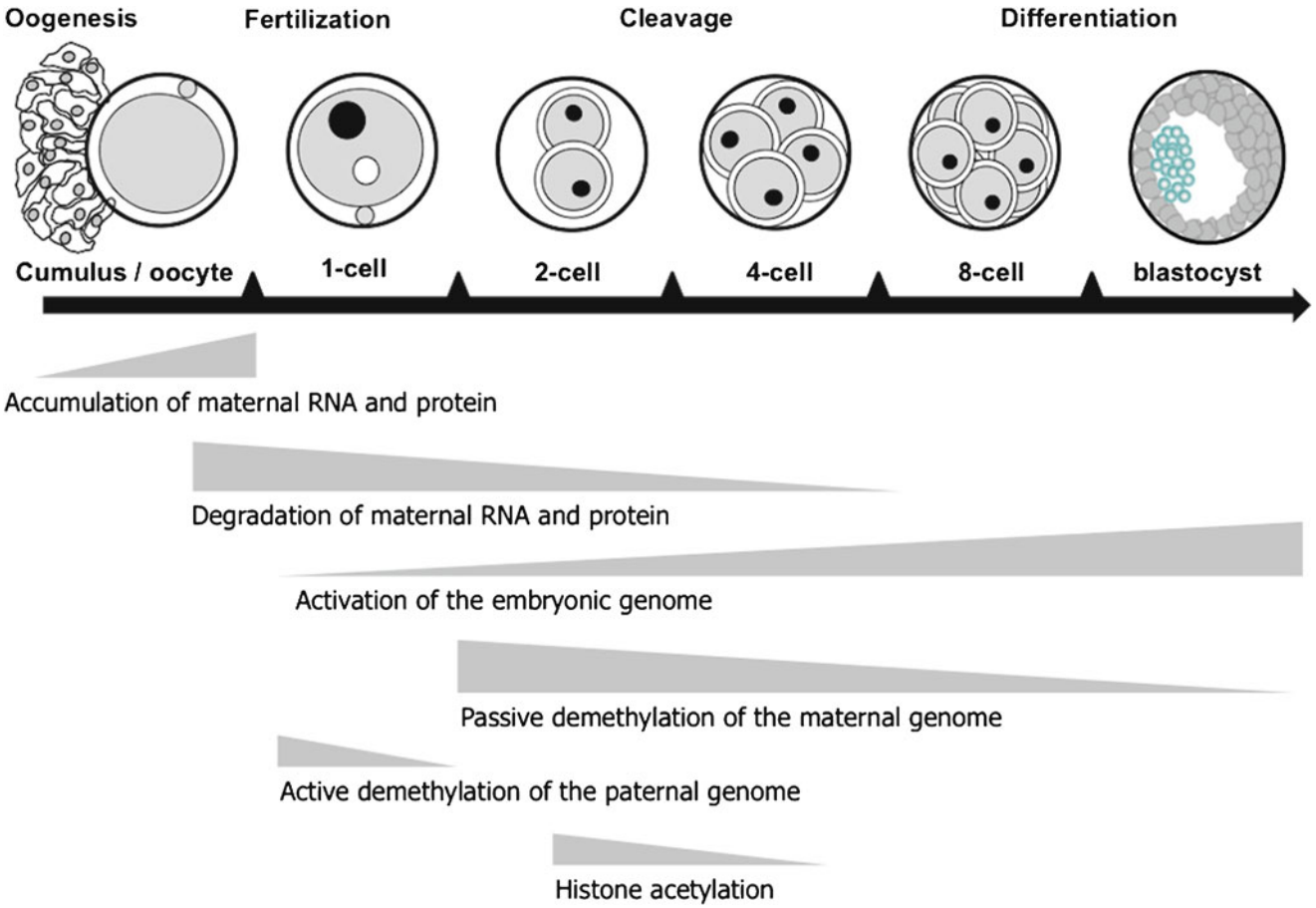

Fig. 46.1 Summary of events in human preimplantation development. Major trends of gene expression changes during early embryonic development. Morphologic transition of preimplantation human embryo and changes in the DNA modification, transcription of maternal and embryonic RNA has been shown at different stages after fertilization

differentiation of the morula into a blastocyst (approximately 64 cells), composed of inner cell mass (ICM) that will give rise to an embryo proper and differentiated cell of the trophectoderm (TE) that will give rise to extra embryonic tissues. ICM contains the cells retaining pluripotency by which they are able to generate three germinal layers: ectoderm, mesoderm, and endoderm. Pluripotent cells can be isolated, adapted, and propagated indefinitely in vitro in an undifferentiated state as human embryonic stem cells (hESCs) [1, 2]. hESCs must retain pluripotency while rapidly proliferating in culture. A group of transcriptional regulatory genes are known to be critical for pluripotency such as *POU5F1/OCT4*, *NANOG*, and *SOX2* [3, 4]. hESCs are remarkable in their ability to generate virtually any cell type, hence carrying many hopes for cell therapy. These cells as well as human mature MII oocytes are both able to achieve the feat of cell reprogramming toward pluripotency, either by somatic cell nuclear transfer or by cell fusion, respectively [5–8]. Comprehending their molecular mechanisms is therefore crucial for better understanding of human development and refinement of assisted reproductive technology (ART) and for regenerative medicine. Significant efforts were made in order to understand the genes expression during the first stages of development until the blastocyst formation. The recent progress in RNA amplification methods and microarray platforms allows us to apply global gene expression profiling to the studies of the oocytes and preimplantation embryos. DNA microarray technology is a major technical breakthrough that can monitor the expression of whole genome within a single experience. This technology provides a unique tool for the determination of gene expression at the level of messenger RNA (mRNA) at a genomic scale. Microarrays have been successfully applied to the high performance expression of many thousands of genes in a single assay. Monitoring changes in gene transcription on a genome-wide basis allows identification of groups or clusters of genes

that are functionally related to a cell or tissue phenotype. This technological capacity has opened new paths for biological investigation and generated a large number of applications [9], including the analysis of early embryo development transcriptomic profile.

Recently, there was an important number of reported papers relate to the use of microarrays in ART program [10–13]. The application of this technology to the analysis of early embryonic development poses specific challenges associated with the picogram levels of mRNA in a single oocyte and embryo, the plasticity of the embryonic transcriptome, the scarcity of the material, and ethical considerations. Microarrays on early embryonic development have allowed us to (1) advance our knowledge of the basic biology of oogenesis, fertilization, and preimplantation embryo development; (2) characterize the gene expression profiles of oocyte; and (3) assess the effect of various ART on global patterns of gene expression in the resulting embryos [14]. This technology also has the potential to identify key molecular biomarkers to assess the quality of oocyte and preimplantation embryo. Finally, early embryonic development offers a relatively homogeneous biological system that is well adapted to expression profiling studies. This chapter highlights the recent advances in elucidating the molecular mechanism complexity in early embryonic development. The knowledge of the global pattern of gene expression is important for understanding critical regulatory pathways that are necessary for early embryonic development. Certain gene expression profiles help to unravel the mystery of these developmental stages. Further understanding of the biological role of these genes may expand our knowledge of the oocyte maturation, fertilization, chromatin remodeling, totipotency, pluripotency, and cell reprogramming. The practical implications of compiling gene expression information on human oocytes and embryos would be enormous since it would enhance the already existing efforts to solve problems related to infertility.

Gene Expression Changes During Oocyte Maturation

Embryo development in mammalian is largely dependent on maternal stored mRNAs and proteins produced during oocyte growth phase [15]. During follicle growth, the nuclear maturation of oocytes is arrested at meiotic prophase-I. Transcriptional activity decreases as the oocyte reaches its maximal size [16]. However, the cytoplasm progresses through a series of maturational stages, accumulating mRNAs and proteins that will enable the oocyte to be fertilized and progress through the first cleavage divisions until embryonic genes begin to be expressed. For improving oocyte maturation, microarray technology permits to assess global gene expression changes of mouse and human oocytes. Therefore, differences in the global mRNA transcript profile have been reported in both mouse and human (1) between mature and immature oocytes [10, 17–20], (2) between younger and older oocytes [21–23], (3) between oocytes developed in vitro or in vivo conditions [24–26], and (4) in oocytes from polycystic ovary syndrome (PCOS) patients [27]. A global transcriptome comparison of germinal vesicle (GV)-stage and mature (MII)-stage mouse oocytes revealed that 2,000 genes were upregulated in MII-stage [17]. The authors reported a higher representation of genes associated with protein metabolism, the mitotic cell cycle, electron transport, fertilization, DNA replication, microtubule/cytoskeletal protein family, G-protein-coupled receptors, and expression signaling in mature oocytes (MII). Similarly, the genome-wide gene expression of human immature (GV and metaphase I [MI]) and mature oocytes (MII) reported minor modification of transcript profiles between GV and MI oocytes, while MII oocytes showed overexpression of more than 400 genes and a striking underexpression of more than 800 genes in comparison with the less matures oocytes [10]. Thus, major modifications of the transcript profile appear to occur during the final stage of the oocyte maturation. Components of the M-phase promoting factor, the anaphase-promoting complex, and a number of oocyte specific genes were overexpressed in MII oocytes [10]. A recent study has indicated that old age alters the expression of mouse oocyte genes involved in oxidative stress, chromatin structure, mitochondrial function, DNA methylation, and genome stability [21]. In addition, the expression profiling of young and old oocytes revealed changes in the expression of several kinetochore components of the spindle assembly checkpoint (SAC), including Cdc20: a critical activator of the anaphase-promoting complex and protein kinases (e.g., Bub1, Aurora kinase) [22]. In human, the expression of genes implicated in stress responses, cell cycle regulation, cytoskeletal structure, energy pathways, and transcription control is affected with age [23]. Recently, a substantial difference between younger and older oocyte at the transcriptional level of genes involved in establishment and organization of the meiotic spindle checkpoint, DNA repair, regulation of meiotic cycle, and cell cycle has been reported by a microarray analysis performed on individual oocytes of advanced age women [28]. Microarray technologies were applied to identify the differences between developments in vitro vs. in vivo. The differences in gene expression detected may assist in the design of optimized protocols for ovarian stimulation and in further refining media formulations for in vitro maturations (IVM). In human, Jones et al. [24] compared the transcriptome of

oocytes matured in vivo (with relatively high developmental competence) with the transcriptome of oocytes under in vitro IVM conditions (with low developmental competence) and identified an overabundance of a large number of genes in oocytes matured in vitro compared with in vivo. This study suggested that the increase in gene expression detected in vitro could be due to either a deregulation in gene transcription or the posttranscriptional modification of genes, causing an inadequate temporal utilization of transcripts which could be translated as a developmental incompetence of any embryos resulting from these oocytes. Wells and Patrizio [26] elegantly differentiated the mRNA expression in human oocytes at different maturational stages under IVM. They observed that high levels of mRNAs, proteins, substrates, and nutrients are accumulated in the oocyte during maturation and are associated with oocyte developmental competence. In addition, IVM seems to alter the abundance of certain MII oocyte mRNAs compared with in vivo MII oocytes [29, 30]. Studies comparing gene expression arrays in tissues from patients with PCOS compared with normal responders have reported similar pathways for differentially expressed genes in whole ovaries [31, 32] and oocytes [27]. Wood's group identified 374 genes with different mRNA transcripts when analyzing morphologically indistinguishable oocytes from ovulatory normal women and from patients with PCOS [27]. Sixty-eight of the differentially expressed genes involved chromosome alignment and segregation; other genes contained putative androgen receptors. These differences could provide a partial explanation for the reduced fecundity observed in PCOS.

Gene Expression Changes During Embryo Development

The early stages of the embryo manifest an autonomous form of developmental resources by different products, which are provided by the oocyte and subsequent from the activation of embryo genome. Despite this autonomy, they are highly sensitive to the environment under in vitro culture condition. The culture condition can influence the gene expression pattern [33]. Comprehensive analysis of gene expression using microarray approach has recently been achieved for minute samples, such as single embryos. The insight gained from such investigations promises to revolutionize the understanding of embryo biology and to identify new molecular biomarkers of embryo quality and abnormal gene expression in embryo with reduced competence. Most studies have examined global gene expression changes in mice [18, 34–36] as well as in human embryos [14, 37–40]. Gene expression profiling of early mouse embryos showed characteristic patterns of maternal RNA depletion and revealed embryonic genome activation (EGA) [41]. Using a cDNA microarray comprised of 6,144 genes, Chen et al. [42] observed global gene expression changes during the hatching of mouse blastocysts, an essential process for implantation [42]. This study determined that 85 genes were upregulated as blastocysts at the hatching stage. These genes included cell adhesion molecules, epigenetic regulators, stress response regulators, and immunoresponse regulators. Dobson et al. [40] studied the global patterns of gene expression in eight human embryos on days 2 and 3 by using DNA microarray analysis. This study revealed that the first few days of embryo development and oocyte maturation were characterized by a significant decrease in transcript levels, suggesting that decay of RNAs associated with gamete identity is integral to embryo development [40]. In addition, this study revealed that developmental arrest and activation of the embryonic genome are unrelated events. Wells et al. [38] used reverse transcription and real-time fluorescent PCR to quantify the expression patterns of nine known genes (*BRCA1*, *BRCA2*, *BUB1*, *RB1*, *ATM*, *APC*, *TP53*, *MAD2*, and *beta-Actin*) at the early stage of human embryos. Their data has suggested the characterization of gene expression profiles for the different stages of early embryo development [38]. Li et al. [39] used a cDNA microarray containing 9,600 transcripts to investigate 631 differential gene expressions in oocytes, as well as the 4-cell and 8-cell human embryo stages. The expression of 184, 29, and 65 genes was found to have a value higher than two times the median value of all genes expressed in oocytes, 4-cell, and 8-cell embryos, respectively, indicating the expression of some zygotic genes had already occurred at the 4-cell embryo stage [39]. Furthermore, Adjaye and Lehrach, by using a cDNA microarray where 15,529 cDNA were spotted, identified biomarker transcripts specific to ICM when comparing gene expression profiles of ICM and trophectoderm cells (TE) from human blastocysts [43]. This data has shown that 1,324 genes were overexpressed at the blastocyst stage, including a proteasome (*PSMA1*, *PSMB3*), cellular trafficking (*SLC2A14*, *SLC1A3*), protein modifications, and turnover (*TNK1*, *UBE3A*). On the other hand, damage to DNA can have severe cellular consequences. These consequences are even worse when the damage occurs in the cells which are the origin of all cells of the future human being. Hence, DNA repair systems play a crucial role in the early embryo development [44]. The importance of DNA repair gene products during development is highlighted by the phenotypes observed for human genetic disorders associated with DNA damage response defects (reviewed in ref. [45]). Recently, DNA repair gene expression was investigated [46] in human oocytes and blastocysts to identify the pathways involved at these stages and detect potential differences in repair mechanisms pre- and postembryonic genome activation. Large numbers of repair genes were detected indicating

that all DNA repair pathways are potentially functional in human oocytes and blastocysts [46]. Finally, transcriptome analysis within the context of embryos can be used to understand a physiological process, mainly done in a research environment with human samples, or it can be used for diagnostic purposes where the goal is to select the best embryo [12, 14, 47].

Comparative Molecular Signature of Metaphase II Oocyte and Inner Cell Mass

Early human embryo development results in the reprogramming of highly specialized germinal cells into pluripotent. The somatic cell can be reprogrammed to a pluripotent state by factors in the oocyte and embryonic stem cells (ESCs) cytoplasm [5, 48]. In this context, the cytoplasmic environments of oocytes and ESC share the capacity to reprogram a somatic nucleus. It is important to determine what events lead to the reprogramming a somatic nuclei after fusion with an oocyte or ESC from the ICM. This issue has been the subject of intense investigation, and studies have focused predominantly on gene expression. The comparison of the gene expression program of these two cell types (oocyte/ESC) could contribute to the understanding of these cell reprogramming properties. To identify these genes, gene expression profiling data of human oocytes and human ES cells were explored [49, 50]. Kocabas et al. [49] compared the transcriptome of human metaphase II oocytes and hESC with reference samples, 1,626 transcripts are significantly upregulated in hESC, and 5,331 transcripts are upregulated in oocytes. The comparison of these transcripts shows that 388 common genes are believed to play an important role in oocyte reprogramming and early events associated with human embryo development. This list of genes includes *POU5F1/OCT4*, *DAZL*, and *DNMT3b* [49]. Assou et al. compared the gene expression profile of mature MII oocytes and hESC and both to somatic tissues [50]. This analysis revealed 652 transcripts conserved in both, oocytes and hESCs, with 1,970 transcripts (2009) detected specifically in the oocyte, in contrast to 1,140 transcripts in the hESCs. These included a significant number of novel genes. The *oocytes signature* comprised *DAZL*, *SOX30*, *ZP2*, *GDF9*, and *AURKC*, which have previously been identified as overexpressed by oocytes [10]. Similarly, the *hESC signature* displayed numerous genes known to be specifically overexpressed in hESC such as *POU5F1/OCT4*, *NANOG*, and *DPPA4*. Genes associated with pluripotency such as *LIN28* and *TDGF1* and a large chromatin remodeling network (*DNMT3B*, *TOP2A*, *JARID2*, *SMARCA5*, *CBX1*, *CBX5*) were found in both oocyte and hESCs. Interestingly, a large set of genes were also found to code for proteins involved in the ubiquitination and proteasome pathway were overexpressed in MII oocytes and hESC. The proteasome is known to interact with chromatin and function at multiple steps in transcription, both through proteolytic and nonproteolytic activities [51]. These results shed light on the molecular mechanisms of early human development and suggest that the proteasome pathway may play a role in initiating and maintaining pluripotency and be used as criteria for oocyte competence. Finally, the comparison of human mature oocytes and hESC helps to understand the early embryo development and pluripotency and is therefore relevant for therapeutics, including improvement of the pregnancy success rate in IVF and regenerative medicine applications such as those involving cell reprogramming.

Apoptotic Events During Embryo Development

The preimplantation period is a critical time for embryo development. More than 50% of the human embryos cultured in vitro fail to reach the blastocyst stage [52]. They exhibit fragmentation and/or arrest their development. Classical apoptotic features have been observed in human and animal preimplantation embryos produced both in vivo and in vitro [52–56]. Apoptosis is a normal mechanism involved in fundamental processes of life, like embryonic development and tissue homeostasis. Detection of apoptosis processes has a big importance for understanding many biological events. It has been proposed that apoptosis may play an important role in eliminating zygotes or embryonic cells carrying chromosomal anomalies or presenting nuclear and/or cytoplasmic defects [57]. However, the degree of apoptosis cannot exceed a certain threshold without leading to the disruption of embryonic homeostasis and developmental arrest. Thus, the balance between pro- and antiapoptotic factors is crucial in determining the fate of the embryo. Bcl-2 family proteins are key regulators of apoptosis; they can be subdivided into two groups: anti- (such as Bcl-2, Bcl-xl, Mcl-1, and Bcl2l10) and proapoptotic (such as Bax, Bak, Bik, and Hrk) members. They act at the mitochondrial level by controlling the release of apoptogenic factors such as cytochrome C, allowing the activation of the downstream executional phase, including the activation of caspases. Transcriptomic analysis revealed a dynamic expression profile of various Bcl-2 family members during early embryonic development (Fig. 46.2) (such as Mcl-1, Bcl2l10, and Bax) [54, 58, 59]. However, the precise role of these proteins in embryo survival needs to be clarified. Caspase 2 and 3 transcripts are expressed at all preimplantation developmental stages [54, 60], and increased caspase 3 activity has been reported in fragmented 4-cell embryos [54]. Finally, survivin plays a key role in embryonic development. It inhibits apoptosis by binding activated caspases (especially caspases 3, 7, and 9). Transgenic embryos, lacking survivin expression, die

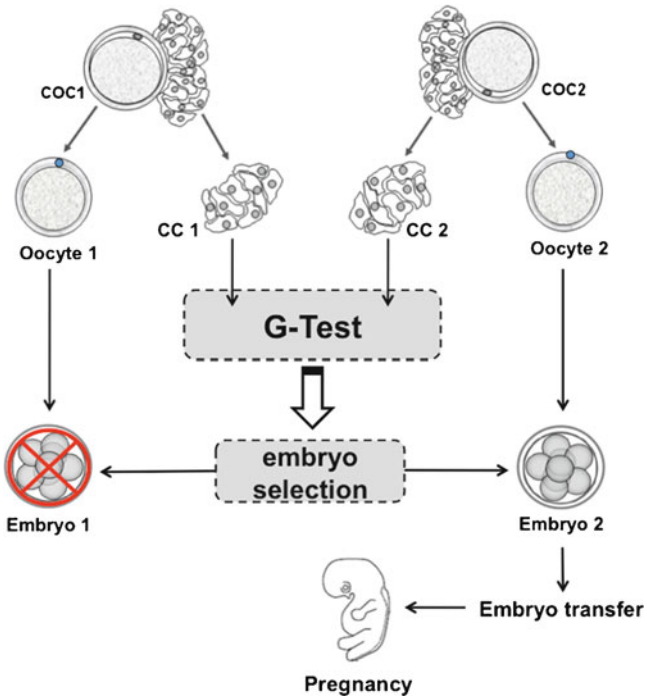

Fig. 46.2 Assessment of embryo quality and pregnancy outcome by *G-test*-based transcriptomic analysis of cumulus cells. *COC* cumulus-oocyte complex; *CC* cumulus cells

in utero between the morula and blastocyst stage due to massive apoptosis [61]. Apoptosis of hESCs is considered to be a dynamic process influenced by the culture conditions. Molecular mechanisms controlling the apoptosis of stem cells are still poorly understood and are only hypothetical.

Epigenetic Event During Early Embryonic Development

DNA methylation is an important epigenetic event, regulating gene expression in any developmental processes including gene imprinting and embryogenesis [62]. The paternal genome is actively demethylated in the few hours after fertilization in human zygote. The mechanism responsible for active demethylation in mammals is currently unknown; however, various DNA repair enzymes have been proposed to accomplish this function [63], while the maternal genome is passively demethylated after the 2-cell embryo stage. To maintain methylated DNA, DNA methyltransferases (DNMTs) are necessary. Recently, our team indicated that *DNMT1* transcript was upregulated in human mature oocytes and decreased progressively in embryos day 3, and in hESC [14], *DNMT3A* was enriched in undifferentiated hESC [4], and *DNMT3B* mRNA was detected in both mature oocyte and hESC [50]. Thus, providing a mechanism for the maintenance of DNA methylation and indicating that the *DNMT3* family methyltransferases are candidate epigenetic regulator during early embryonic development. Imprinted genes are genes whose expression is determined by the parent that contributed them. The majority of imprinted genes have been found to have roles in the control of embryonic development [64]. Studies made have established that genetic imprinting is highly conserved among mammals. As imprinting is related to early embryonic development, our team analyzed the expression of 40 imprinted genes and revealed that distinct sets of imprinting genes appear to be involved in human early embryonic development [14]. The imprinted genes can offer new insights into the mechanisms of gene regulation. Gene imprinting provides a form of control of a gene expression. The study of the epigenome is important because minor defects can lead to serious human diseases. Indeed, several syndromes are associated with imprinting defects during early embryonic development [65]. In addition, not only are genes encoding proteins involved in the control and regulation of early embryonic development but also small noncoding RNAs such as microRNAs (miRNAs) that function to regulate gene expression [66]. MiRNAs are approximately 22 nucleotides in length. These are thought to function as repressors of gene expression by blocking mRNA translation [67]. Their normal activities help to coordinate multicellular life, and their dysregulation can contribute to a variety of diseases. The importance of miRNAs regulatory roles is underscored by literature evidence that miRNA function is involved in almost all biological processes, including stem cell regulation [68], cancers [69], and others. The expression patterns of miRNA have been exploited as a new class of biomarkers for disease phenotypes and tissue classifications [67]. To date, the global effect of miRNA during early embryonic development is still undiscovered.

Clinical Applications for Human Embryo Selection

Our understanding of molecular determinants of embryo viability is limited; consequently, current embryo assessment strategies in clinical settings largely rely on embryo morphology. However, most studies suggest that embryos with proper morphological appearance alone are not sufficient to predict a successful implantation. Considering the limitation of morphologic evaluation, there is now a movement toward more sophisticated technologies, such as microarray. This method, involve the analysis of whole embryos, represents a valuable approach to biomarker development, rendering them useless for purposes of clinical testing. Both technical and statistical advances are currently facilitating the application of this approach in ART program. Microarray technology has been used experimentally in humans from

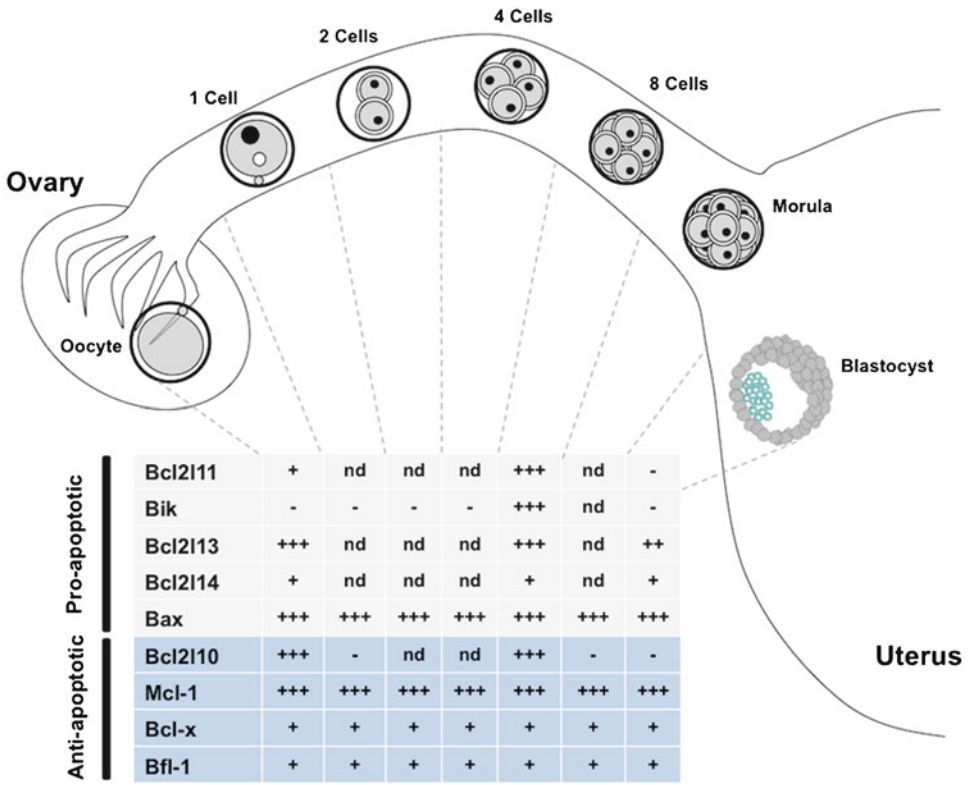

Fig. 46.3 Expression of Bcl-2 family members during early embryonic development. Early embryogenesis stages are presented schematically. The expression levels of the different members are relative, derived from our data and the synthesis of several studies. Very high (+++), high (++), medium (+), low (−), not detected (nd)

trophoblastic biopsies performed on blastocysts. This makes it possible to identify candidate genes involved in adherence or cellular communication with a level expression related to implantation [47]. An indirect and attractive approach for predicting embryo quality and pregnancy outcome has been recently reported by using transcriptomic data of cumulus cells (CCs) [11, 12]. This approach is noninvasive based on the major role of the dialogue between oocyte and surrounding CCs during oocyte maturation. Oocytes and CCs communicate in the follicles through locally secreted factors and neuroendocrine signals. Many studies provide evidence [11, 70] that CCs (1) coordinate follicle development with oocyte maturation, (2) provide energy substrate for oocyte meiotic resumption, (3) regulate oocyte transcription, (4) promote nuclear and oocyte molecular maturation, (5) stimulate amino acid transport and sterol biosynthesis, (6) promote glycolysis [71], and (7) protect the oocyte from the harsh environment. The CCs metabolize alternative substrates, such as amino acids, cholesterol, and glucose, which are essential for the development and function of oocyte [72–74]. CCs share the same follicular environment as the oocyte and both control early as well as delay follicular development. In addition, oocyte quality is a major determinant of the embryo's developmental capacity. Thus, analysis of gene expression in CCs may provide an undirected indication of the microenvironment in which the oocyte matured and will help biologists to achieve a better capability of selecting embryos competent for transfer. Since CCs can be obtained easily during intracytoplasmic sperm injection (ICSI) procedure, and regarding the physiological relevance of the oocyte-CC dialogue during the oocyte maturation process, several groups used microarrays technologies, RT-PCR and quantitative RT-PCR analyses to associate CCs gene expression profile with oocyte competence [75, 76], embryo quality [75–77], and pregnancy outcome [11, 12, 78]. By comparing gene expression profiles of CCs according to embryonic quality and pregnancy outcome, our group reported for the first time a specific transcriptomic signature including 630 genes associated with pregnancy outcome. The majority of the genes differentially expressed were upregulated, suggesting that transcriptional activation in CCs is essential to acquire embryonic competence. Interestingly, genes in CCs resulting in a successful pregnancy were predominantly upregulated, including *BCL2L11* and *PCK1*, respectively involved in apoptosis and gluconeogenesis. Some genes were nevertheless found down regulated in CCs associated with a good pregnancy outcome, such as the transcription factor *NFIB* [12]. In addition, we developed a new potential method "genomic test (G-test)" for competent embryo selection, comprising a step of measuring the expression level of potential biomarkers in CCs (Fig. 46.3). Recently, we conducted a prospective study

including 45 biomarkers in CCs associated with embryo and pregnancy outcomes and young (<36 years) normal responder patients referred to our center for cIVF or for ICSI. The embryo selection occurred either according to biomarker profile in CCs (group 1) or to morphological aspects (group 2 used as control). In each group, two embryos were replaced. Our preliminary data of embryo outcome shows the absence of relationship between morphological aspects of embryo and gene expression profiles in CCs [11]. It is time to reconsider the notion that embryos presenting a low grade according to morphological aspects are able to achieve pregnancy. To date, several of our biomarkers are actually validated in larger clinical trials to assess their predictive value. We have established a mathematical model to identify embryo with high implantation potential. Additionally, measurement of nutrients and metabolites extracted into media surrounding the embryo provides information concerning viability. A multitude of studies proposed that the change in the levels of their metabolites would reflect embryo viability and assessed these metabolites as potential biomarkers [79, 80]. Other studies showed that metabolomic profiling of embryo culture media was independent of morphology, providing an independent parameter in embryo viability assessment [81, 82]. Finally, transcriptomics on CCs and metabolomics on culture media techniques are noninvasive, and once adopted, they will allow us to maximize the success of IVF treatment options and should improve in the near future the embryo selection for fresh replacement that is based in part on morphological criteria. Each technique is powerful in itself, but the combination of these techniques will probably revolutionize the success of IVF.

Conclusions

Knowledge of the global pattern of gene expression is important for understanding critical regulatory pathways and key biological process governing human reproduction such as oocyte maturation, embryo development, and ESCs biology. Many studies provided evidence that genes involved with intracellular signaling, cell survival/death, pluripotency, transcription and translation, methylation/demethylation, and nuclear reprogramming are the main cellular processes that control the early embryonic development [14]. In this chapter, we did try to relate for readers the recent advances about the dynamic genes changes of human early embryo development and the application of DNA microarray as a new emergent technology for embryo selection. The use of gene expression of CCs as biomarkers can predict indirectly embryo quality and pregnancy outcome. Investigations on biomarkers offer new and exciting opportunities for embryologists with bioengineering expertise. Techniques of this type may ultimately lead to noninvasive tests for oocyte or embryo quality, revealing previously hidden information concerning both oocyte and embryonic developmental competence. This chapter will be great to those interested on early embryo development; these findings are relevant for therapeutics, including improving the regenerative medicine applications involving pluripotent cells (hESC). Most importantly, our increased knowledge of genes and related transcriptional networks operative during early embryonic development should lead to advances in ART and preimplantation genetic diagnosis.

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Amino Acid Turnover as a Biomarker of Embryo Viability

47

Christine Leary, Danielle G. Smith, Henry J. Leese, and Roger G. Sturmey

Abstract

Selection of the most viable embryo to transfer and at which stage of development remains one of the most challenging aspects of in vitro fertilization. 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. 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 the introduction of external quality assessment schemes, 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. 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. Amino acid profiling differs from conventional metabolic assays, where typically, only one or two metabolites are measured.

Keywords

Biomarkers of embryo viability • Early embryo metabolism • High performance liquid chromatography • Tools for single embryo transfer

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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 postinsemination on the basis of the embryos cleavage rate and morphological appearance. Studies have shown that correlations exist

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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 with implantation include blastomere size, 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 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.

Why the Need for Objective Markers of Viability?

Embryo selection is currently based on operator observations of embryo developmental and morphology 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 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.

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 1–2 or day 2–3 postinsemination.

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.

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.

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 two major consumers of energy, the other being the Na+, K+, ATPase; their contributions to the energy budget being ~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 methodology and assumptions. Despite these caveats, it is most likely that the protein synthesis will be a major component of energy homeostasis and, in the context of the present discussion, provide a good marker of the overall nutritional requirements of the early embryo.

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] where in addition to providing purine and pyrimidine nucleotide precursors it acts as an energy source.

Osmolytes

Notable among amino acids which act as osmolytes during mouse preimplantation development are glycine, betaine, glutamine, and proline [26].

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.

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.

How to Measure Amino Acids

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 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.

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 and 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 therefore slightly less hydrophobicity 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 r*everse phase* HPLC, distinguished from conventional HPLC because the mobile rather than solid phase in the column is charged (Fig. 47.1). 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 *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 the hydrophobic amino acids, such at leucine.

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 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 dependant. 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. 47.2.

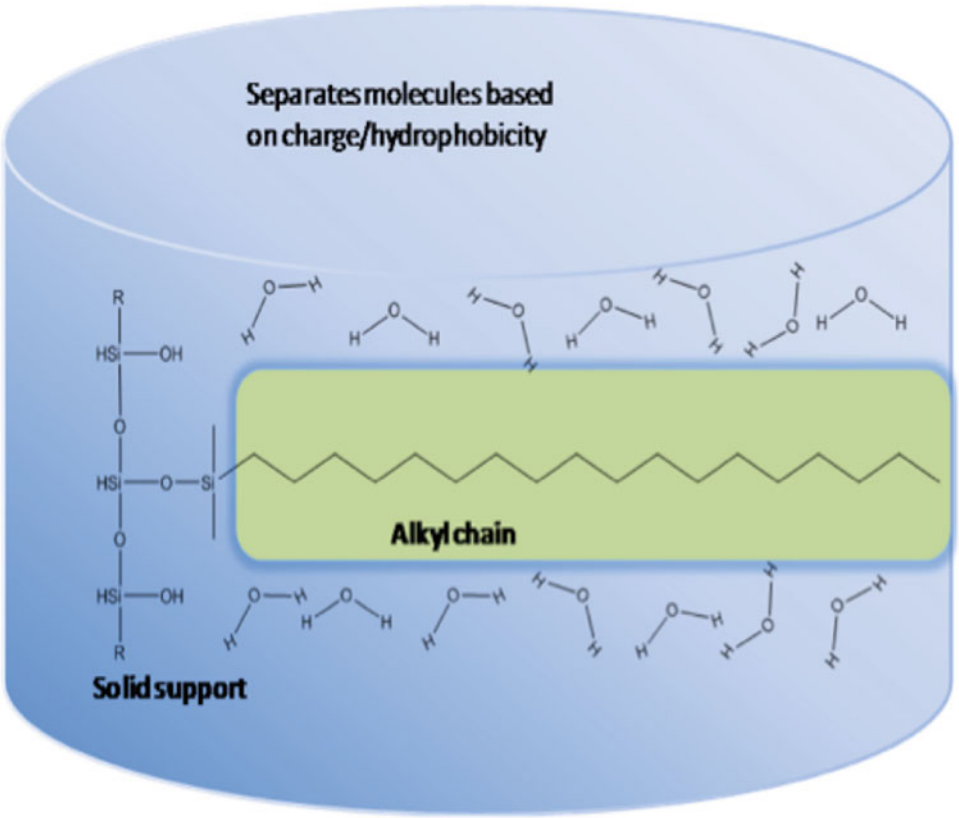

Fig. 47.1 Reverse phase column. The solid support of the column is usually made of chemically inert material of a large pH range, for example, silica. Attached to the inert core are hydrophobic fingers (*green*) made of up alkyl chains. Hydrophillic pockets are formed between the alkyl chains as the 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

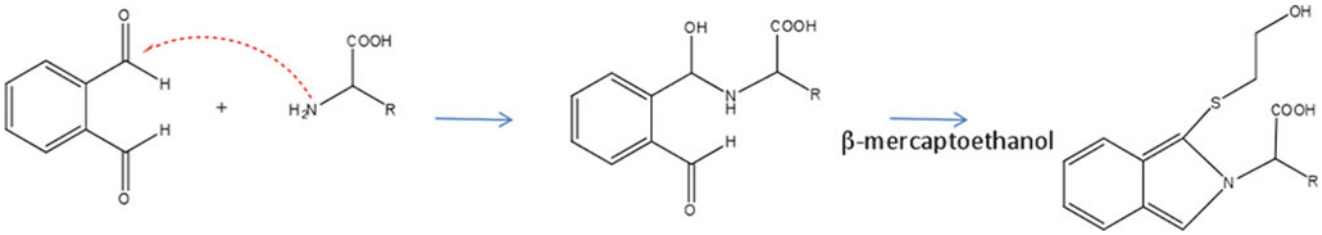

Fig. 47.2 Amino acid derivatization with OPA. The carbonyl of the OPA undergoes nucleophilic attack by the nitrogen of the amine of an amino acid generating intermediate B. In the presence of mercaptoethanol, intermediate B will rapidly convert to the fluorescently detectable isoindole product C

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 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 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.

How to Prepare Samples and *Do* the 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 an 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.

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.

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 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 the 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.

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 outcome [16]. However, in order to draw further conclusions from these types of approaches, full randomized prospective clinical trials are now required.

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Real-Time Embryo Monitoring Device for Embryo Selection

48

Gábor Vajta and Thorir Hardarson

Abstract

Noninvasive methods for evaluation of the quality and prediction of developmental competence of in vitro-produced preimplantation-stage embryos are of crucial importance in human-assisted reproduction. Currently, one of the most demanding tasks in this field is to find the optimal solution between two partially contradicting requirements: to achieve the highest pregnancy (and live birth) rates and to keep the proportion of multiple pregnancies at a minimum level. The most feasible approach to meet these requirements is the extended culture and decreased number of transferred embryos; the former may result in the survival of the best embryos, while the latter decreases the chance of multiple implantation. The ultimate realization of this strategy is the single blastocyst transfer. At present, the only routine method for this prediction and selection is the bi-daily light microscopic evaluation of the morphology. The limits of this approach are widely acknowledged and are related to the snapshot type analysis of dynamic processes. Alternate possibilities include specific investigation of certain structures or combined evaluation of morphological features, and sophisticated indirect analysis of intracellular processes by measuring changes in certain parameters in surrounding media. A third approach is the real-time light microscopic monitoring of embryo development by using time-lapse techniques.

Keywords

Real-time embryo monitoring • Embryo monitoring • Noninvasive embryo monitoring • Time-lapse techniques in mammalian embryology • Video monitoring of embryos

Noninvasive methods for evaluation of the quality and prediction of developmental competence of in vitro-produced preimplantation-stage embryos are of crucial importance in human-assisted reproduction. Currently, one of the most demanding tasks in this field is to find the optimal solution between two partially contradicting requirements: to achieve the highest pregnancy (and live birth) rates and to keep the proportion of multiple pregnancies at a minimum level. The most feasible approach to meet these requirements is the extended culture and decreased number of transferred embryos; the former may result in the survival of the best embryos, while the latter decreases the chance of multiple implantation [1]. The ultimate realization of this strategy is the single blastocyst transfer [2–4]. Former concerns related to the compromised cryosurvival of blastocysts and decreased chances of a subsequent cryocycle have been mostly eliminated with the introduction of highly efficient vitrification techniques [5–7].

This strategy, however, is not always applicable to all patients. Although conditions of embryo culture have improved considerably, they are still regarded as handicapped compared to the in vivo situation. Accordingly, the

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best place for preimplantation development is the maternal oviduct and uterus, and extended culture may endanger the success of the assisted reproductive cycle, especially when the number of available oocytes is limited and/or the development at the early cleavage stages is compromised. The growing application of minimal stimulation may result in developmentally more competent oocytes and more appropriate in vivo environment for implantation, but decreases the number of obtainable oocytes. Accordingly, the decision regarding the most optimal timing of embryo transfer may become even more complicated.

At present, the only routine method for this prediction and selection is the bi-daily light microscopic evaluation of the morphology. The limits of this approach are widely acknowledged and are related to the snapshot type analysis of dynamic processes [8, 9]. Accordingly, there is an increasing need for alternative methods. Possibilities include specific investigation of certain structures or combined evaluation of morphological features, and sophisticated indirect analysis of intracellular processes by measuring changes in certain parameters in surrounding media [10–16]. A third approach is the real-time light microscopic monitoring of embryo development by using time-lapse photography.

Development of Methods for Time-Lapse Investigations

The idea to apply time-lapse techniques in mammalian embryology is almost as old as the successful embryo culture itself. The first time-lapse investigations on preimplantation embryos were performed in rabbits as early as in 1929 by Lewis and Gregory, followed by investigations in mice [17–19] and in cattle [20, 21], with the purpose to observe some changes in structure elements or to analyze mechanism of dynamic processes including expansion and hatching. These studies were extended later to measurements of cell cycle length, investigation of compaction, blastocoel formation, and compare the effects of various culture conditions [22, 23]. As a more complex task, time-lapse observations were also used to predict developmental competence and sex in golden hamster and mouse embryos [24, 25]. In humans, oocyte activation, pronuclear formation, and cleavage after intracytoplasmic sperm injection were studied with this technology [16]. Time-lapse investigation has helped to understand the mechanism of polar body extrusion and pronuclear formation [26] and provided a direct evidence about the reversible nature of fragmentations [7, 27]. Early disappearance of pronuclei and onset of the first cleavage were found to be correlated with a higher number of blastomeres on Day 2 after oocyte retrieval, proving the predictive potential of time-lapse investigations regarding early-stage development of human embryos [28].

In spite of the demonstrated value of this technique, so far investigations were restricted to the experimental level, and application in an unselected cohort of patients in a routine infertility program was not reported due to the high costs, complicated structure, and the limited capacity of available equipments. Almost all previous studies have used individually designed or specially modified systems; commercial production of various time-lapse equipment has started only recently. However, the principle was the same at all: either to build up an incubator over the stage of a commercially available inverted microscope or to put a modified microscope into a commercially available incubator.

Requirements for a Time-Lapse System for Routine Application in a Standard Human Infertility Program

According to our present knowledge, a real-time light microscopic system to monitor preimplantation-stage human embryo development should meet the following criteria:

1. Provide an optically satisfactory picture suitable for safe diagnosis of key milestones and important features of preimplantation development including the size and shape of the embryo; the intactness, density, and approximate thickness of the zona pellucida; the size of the perivitelline space; intactness of the oolemma; cytoplasmic abnormalities (color, vacuoles, inclusions, granulations), formation, and number of pronuclei; cleavages; number and size of blastomeres; compaction; blastulation; size and shape of the inner cell mass; morphology of the trophectodermal layer; expansion; and hatching.
2. Enable individual identification of embryos, while monitoring in parallel at least 12–16 embryos (in most cycles, this is enough to control the development of all intact zygotes from one patient).
3. Make pictures with a reasonable frequency (the usually applied and acknowledged parameter is 4–6 pictures per hour) during the whole preimplantation period (0–6 days).
4. Due to the commonly used culture systems, enable medium changes and safe repositioning without mixing up embryos. The removal and replacement of dishes as well as the medium change itself should not be more demanding than the present routine in ART laboratories.
5. At any time point, provide pictures about the actual state of all embryos and all previous events without disturbing the safe incubation and the monitoring process. Optimally, this information should be accessible remotely by the internet for selected staff.
6. Assemble pictures into time-lapse videos, enable morphometric analysis of individual pictures, and archive files with the documentations of patients.

7. Eventually, software may also be added for automatic analysis of measurable parameters, to create quality scores of individual features or the whole embryo.
8. The real-time monitoring system should be free of any measurable or potential harm that may implement more dangers to the embryos than the routine acknowledged embryo culture procedure of the accredited ART unit.

The latter primum nil nocere (first, do not harm—Latin) principle is the basis of all medical interventions and is a key condition when new methods are introduced, even if it is an in vitro procedure and is related to preimplantation-stage embryos. Unfortunately, as also applicable to most diagnostic and therapeutic procedures, it is very difficult to meet strictly this requirement in the ART laboratory. However, efforts should be made to find the optimal solution to keep the unavoidable risks at the minimum level, and the potential benefits should highly outbalance the accidental negative effects that may occur. As real-time monitoring is not indispensable for a successful routine ART program, and its potential benefits are not supported with convincing evidences, any risks related to its application should be seriously considered and eliminated or radically minimized.

Potential sources of damage may include the following:

Frequent exposition to visible light: For most embryologist, this is the primary concern, although this factor may be controlled relatively easily. The sensitivity of mammalian embryos to light is a widely acknowledged fact, although the consensus regarding the quantitative characteristics of the harmful dose including intensity, length, and wavelength is still missing [29, 30]. Some exposition to visible light is part of the routine handling of oocytes and embryos in ART procedures both under the microscopes and in the laboratory room. A safe limit for real-time monitoring may be a maximum 10% increase in the cumulative dose, and the potential damage should also be reduced by using longer, supposedly less harmful wavelengths. These goals are relatively easy to achieve with low-intensity light, appropriate filters, and limiting illumination strictly to the period of exposition.

Physical, chemical, and biological damages related to the applied instruments: Theoretically, a stationary microscope equipped with a camera should not mean a major risk to embryo development. However, this arrangement is insufficient for a routine human infertility program where parallel investigation of different dishes, each one containing the embryos of a single patient is required. The most demanding task was to make the real-time monitoring instruments capable for parallel investigation of multiple embryos, or groups of embryos with a safe individual identification possibility for comparative evaluation.

Sophisticated automatic forwarding and focusing systems were installed both for the "incubator on the microscope" or the "microscope in the incubator" design. Unfortunately, even with the latest technical solutions, systems require complicated mechanical arrangements and advanced electronic regulations. Loading of dishes may be more demanding than placing them on the shelves of the incubator. Accordingly, this may increase the inconsistence of temperature and gas atmosphere of all investigated dishes. Additional heat from motors, electrosmog, volatile compounds of lubricants, seals, and other applied material may also cause detectable or hidden but existing damage on embryos. Sterilization and especially maintenance of sterility of these instruments is difficult or impossible; accordingly, the risk of infection is increased and, once occurs, it may hamper further investigations for long. Moreover, the required constant movement of dishes, and the consequent shear stress may compromise the development [31].

Potential risk of malfunction or breakdown: Optimally, routine application of real-time monitoring systems for human embryos would need the unachievable 100% stability or warranty that an accident occurring in the mechanical or electronic part does not make any harm in the development of observed embryos. This requirement is very difficult to fulfill. Even in commercially available carbon dioxide incubators hidden cable contact problems and related minuscule fumes may result in compromised development without detectable malfunction for months (Vajta, unpublished). The probability of such accidents increases in parallel with the complexity of instruments, number of cable contacts, motors, and moving parts.

Potential Benefits of Real-Time Video Monitoring of Embryos

As none of the available technical solutions can entirely fulfill all technical requirements including elimination of potential dangers, the benefits of application of real-time monitoring in routine human infertility programs should exceed the curiosity of professionals and patients and commercial interests by advertising services with live images from the very first moments of life of the future child. As mentioned earlier, determination of the optimal time point of transfer and selection of the developmentally most competent embryo may justify the application. However, no prospective randomized trial has been performed to investigate if using the time-lapse technique may be an independent predictor of birth. Until such trials are published, the use of the time-lapse technique remains only promising.

Time-lapse investigation may provide more precise data about the morphology and developmental kinetics than the routine bi-daily microscopic control that was reported insufficient for accurate evaluation [8, 9, 25, 28]. Some previous studies have demonstrated strong correlation between short initial cleavage cycles and the subsequent development potential of

individual embryos. [31–42], although other studies alerted that extremely rapidly cleaving embryos in humans may be potentially compromised because of inappropriate imprinting and chromosomal abnormalities [43–46]. These data indicate that the more accurate the determination of the time points of cleavages, the better prediction of embryo development is possible. According to a recent investigation in mice, the most important are the first and second cleavage (to the two- and three-cell stage), respectively, while the time point of the third cleavage (to the four-cell stage) is indifferent [47].

Another crucial feature is fragmentation that may dramatically reduce pregnancy rates [48, 49], and transfer of fragmented human embryos was reported to increase rates of malformations [50]. However, in contrast to the common opinion, fragmentation is a reversible process and a fragment may disappear by resorption as rapidly as within 10 min [7], although the reabsorption does not modify the compromised developmental competence of these embryos. The chance for a fragmented mouse embryo to reach the blastocyst stage might be reduced by up to 60%, compared to non-fragmented embryos, but the average duration for fragmentation and reabsorption was only 9.1 h; accordingly, 36 or 73% of these fragmentations would not had been noticed in daily or bi-daily monitoring [47].

Real-time investigations may also be used as overall quality control measures for the actual level of embryo production of the IVF unit and may also provide early alarm of any extraordinary events that happen inside the incubators or culture dishes including infection, toxic damage of embryos, or other harmful events that are not detected by sensors or routine quality control investigations.

Finally, real-time investigations accompanied with the use of single media without medium change may eliminate the need of routine bi-daily removing of dishes from the incubator. Although most producers still suggest medium change on Day 3, routinely used highly efficient culture systems in large domestic species [51] indicate that the medium change may not be indispensable, and its disadvantages may outnumber the benefits. Slowly increasing number of human data also proves the feasibility of this approach [47]. With the application of routine time-lapse video monitoring, embryos may remain for 4 or 5 days undisturbed in the strictly regulated environments of the incubators that—according to our present knowledge—provides them optimal conditions for preimplantation development in vitro.

Latest Technical Solutions for Real-Time Monitoring

Recent devices produced individually or industrially are mostly based on the earlier principles and consist of either an incubator build above a microscope, or a microscope placed into an incubator. Variations of the former solutions included a small flat gassed and heated incubator chamber, a large box surrounding most part of the microscope, or the combined application of the two to provide even higher stability ([7, 27, 51–54]; Stage-top Incubator, Tokai-hit, Japan; references have been selected only for illustration and without any commercial or other suggestions or preference). To avoid extensive light exposition from the laboratory environment, some of these boxes have been made from nontransparent material.

No problems with the ambient light occurs when the microscope/camera is placed inside the incubator, but commercially available microscopes are not designed for this purpose and require modifications and additional equipment for applicability [25] (InCu-Cell Live, Sanyo, Japan; BioStation, Nikon, Japan). Some of the latest solutions combine the two approaches: a purpose-built incubator accompanied by a purpose-built microscope (Embryoscope, Unisense FertiliTech A/S, Denmark). Typically, these devices are equipped with turntables or other mechanical devices powered by electric motors to enable investigation of multiple embryos, and require also individual focusing before each photograph. Consequently, there is a possibility to expose embryos to shear stress, heat, lubricant fumes, and electromagnetic fields, and the complicated structure may also mean an increased risk of infection.

Another approach for the "microscope into the incubator" principle is a simplified compact digital inverted microscope designed to observe all embryos of a patient in one view field, by using the Well-of-the-Well culture system to locate safely the embryos (Primo-Vision, Cryo-Innovation Ltd, Hungary). The small, sealed digital microscopes are placed inside the regular incubators. The transfer of the images and the control of the microscope units are managed by a PC software through an electric controlling unit that is located outside the incubator and connected to the microscope with a shielded firewire cable through the factory-made side port of the incubator. All electricity is switched off completely in between the image acquisitions, so no potentially harmful continuous electromagnetic effects are present. With the simplified construction and lack of movement of dishes during observation, the dangers mentioned above are eliminated, and an accidental breakdown would not influence at all the normal culture environment. The system has proved its value for prediction of in vitro developmental competence of mouse embryos [52], and the first human pregnancy after continuous monitoring to the blastocyst stage has also been achieved [55].

Future applications of time-lapse equipment may include observation of embryo development in new culture systems including microfluidic-microchannel devices that will allow for media change/gradient coupled with the possibility of analyzing the spent culture media using advanced diagnostic techniques.

Another potential advancement may include automatic analysis of several patters including cleavages, size of blastomeres, fragmentation, or other features with potential predictive value. Some time-lapse devices are already equipped with programs that are capable for limited automatic evaluations. Further development of these softwares and their widespread application may considerably increase the predictive value regarding implantation potential and full-term development.

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Assisted Hatching in IVF

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Itziar Belil and Anna Veiga

Abstract

Blastocyst hatching failure, due to intrinsic abnormalities in either the blastocyst or zona pellucida (ZP) of in vitro cultured embryos, may be one limiting factor in human ART efficiency. Assisted hatching (AH) involves the artificial thinning or breaching of the ZP and has been proposed to improve implantation rates in selected patients following IVF. AH procedure can be performed using various methods. These include the creation of an opening in the ZP either by drilling with acid Tyrode's solution, mechanical partial zona dissection, or laser photoablation. The ZP can be thinned using proteolytic enzymes, acid Tyrode's solution, or laser and totally removed by chemical and enzymatical action. AH is usually performed on day 3 after fertilization but also at the blastocyst stage. Outcome of frozen–thawed cycles can also be improved after AH. AH may be associated with specific complications, including damage to the embryo or to individual blastomeres with reduction of embryo viability and an increased risk of monozygotic twinning due to the artificial manipulation of the ZP.

Keywords

IVF • Zona pellucida • Micromanipulation • Assisted hatching • Partial zona dissection • Laser photoablation • Controlled zona dissection

The Zona Pellucida and Embryo Hatching

Zona Pellucida

The zona pellucida (ZP) of mammalian eggs and embryos is an acellular matrix composed of sulfated glycoproteins with different roles during fertilization and embryo development [1]. Three distinct glycoproteins have been described both in mice and in humans (ZP1, ZP2, and ZP3) [2]. Acrosome-reacted spermatozoa bind to ZP receptors, and biochemical changes have been observed after fertilization [3] responsible for the prevention of polyspermic fertilization.

The main function of the ZP after fertilization is the protection of the embryo and the maintenance of its integrity [4]. It has been postulated that blastomeres may be weakly connected and that the ZP is needed during the migration of embryos through the reproductive tract to maintain the embryo structure. Implantation has been observed after replacement of zona-free mouse morulas or blastocysts, while the transfer of zona-free precompacted embryos results in the adherence of transferred embryos to the oviductal walls or to one another. A possible protective role against hostile uterine factors has also been described [4]. Degeneration of sheep eggs after complete or partial ZP removal that could be ascribed to an immune response was described by Trounson and Moore [5].

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Hatching

Embryo hatching involves a spontaneous rupture of the ZP. Once in the uterus, the blastocysts must get out of the ZP to allow interaction between trophectoderm and endometrial cells so that implantation can occur. The loss of the ZP in the uterus is the result of embryonic and uterine events. Expansion and ZP thinning occurs in mammalian blastocysts prior to hatching. As a result of several cycles of contraction and expansion and because of its elasticity, the ZP thins. Lysins of embryonic and/or uterine origin are also involved in ZP thinning and hatching. It seems that hatching is predominantly the result of zona lysis, whereas physical expansion of the blastocyst, even though involved in hatching, does not seem to be the primary mechanism [6, 7]. Contraction-expansion cycles as well as cytoplasmic extensions of trophectoderm (trophectoderm projections, TEPs) have been documented by time-lapse video recording in human blastocysts [8]. It is not clear whether TEPs are needed in vivo for ZP hatching, but they seem to have a role in attachment, implantation, and possibly embryo locomotion [9]. Mouse embryo hatching in vitro seems to be dependent on a sufficient number of blastomeres constituting the embryo. Hatching in vivo must be different from that in vitro, the difference possibly involving uterine and/or uterine-induced trophectoderm lytic factors [10].

Assisted Hatching

Definition

Failure of implantation after IVF may result from the inability of the blastocyst to hatch out of the ZP. Artificial disruption of the ZP by micromanipulation techniques to enhance the ability of embryo to hatch is known as assisted hatching (AH) and has been proposed as a method for improving the success of IVF. The first report of the use of AH in human embryos was published by Cohen et al. in 1990 [11]. These authors documented an important increase of implantation rates with mechanical AH in embryos from unselected IVF patients.

Indications for Assisted Hatching

Human embryos resulting from superovulation develop more slowly in vitro compared to embryos in vivo, manifest a relatively high degree of genetic abnormalities, undergo cell fragmentation, and only a small proportion achieve blastocyst stage development [12]. Cultured embryos also hatch and implant at lower rates than natural [13, 14]. It is unclear whether this is due to hardening of the zona pellucida as a result of cross-linking of its constituent glycoproteins (ZP1, ZP2, ZP3) in an in vitro environment[4], but it is believed that ZP hardening may be exacerbated at any stage of embryo development after long-term in vitro culture and cryopreservation [15]. Zona thickness appears to be influenced by woman's age and proliferative phase follicle-stimulating hormone (FSH) profile, and correlates negatively with embryo implantation rates [16].

Under these considerations, AH can be performed in the following cases:

- Recurrent failure of embryo implantation after embryo transfer (three or more embryo transfers without a pregnancy)
- Embryos exhibiting thick zona pellucida
- Advanced maternal age (over 37 years)
- Women with elevated FSH levels
- Cryopreservation cycles

How to Perform Assisted Hatching

Embryo implantation seems to occur earlier after AH, possibly by allowing earlier embryo–endometrium contact [17]. Partial ZP drilling, ZP thinning, and total ZP removal are different ways to perform embryo AH using various micromanipulation techniques. It is very important to minimize the time that the embryo is out of the incubator and to optimize methodologies to reduce pH and temperature variations that can be detrimental for embryo development, during embryo manipulation. To reduce environmental variations, AH has to be performed in microdrops of HEPES-buffered medium (to minimize pH changes during the procedure) covered with oil, under an inverted microscope with Nomarski or Hoffman optics, on a heated microscope stage, at 37°C. Microtools for AH can be made by means of a pipette puller and microforge, but are also commercially available. Micropipettes are mounted on micromanipulators. A 30-min culture period seems to be sufficient before the transfer of the manipulated embryos. Embryo transfer to the uterus has to be performed as atraumatically as possible to avoid damage of ZP-manipulated embryos.

Partial Zona Drilling

An artificial gap drilling completely the ZP can be made using micromanipulation techniques. It is important that the size of the hole created in the zona is large enough to avoid trapping of the embryo during hatching, but not so large that it permits blastomere loss. The adequate size of the hole seems to be ~30 mm. Zona drilling can be achieved by mechanical, enzymatic, chemical, or thermolytic effect [18].

Zona Pellucida Thinning

The human zona is bilayered, with a less dense, thick, easily digestible outer layer, and a more compact but resilient inner

layer [19]. The aim of ZP thinning is to thin the outer layer of the ZP without complete lysis and perforation of the inner layer. It involves the thinning of about 50% of the zona thickness of a cross-shaped area (partial zona thinning) or of the complete surface (circumferential zona thinning) of the ZP of the embryo [18]. Care has to be taken not to rupture the ZP completely. By not breaching the zona, the potential risk of blastomere loss and embryonic infection is minimized. Zona-thinned embryos show higher implantation rate and seem to be more physiological than total ZP drilling when embryo transfer is performed on day 3 of embryo culture [20].

Zona pellucida thinning has been described using chemical or enzymatic action or with laser methodology.

Zona Pellucida Removal

Total ZP removal before embryo transfer has been expected to bring about closer contact and communication of the trophectoderm of the blastocyst with the endometrium thereby improving implantation. Full ZP removal has shown to improve the outcome of blastocyst transfer in patients showing repeated implantation failure [21] as well as of blastocyst transfer after vitrification [22]. Total ZP removal at the cleavage stage may have adverse effects such as loss of blastomeres and an increased risk of monozygotic multiple gestation. The ZP can be totally removed by chemical or enzymatical methods or by using laser and mechanical pipetting.

Methods of Assisted Hatching

The AH procedure to breach or thin the ZP can be performed using various methods.

Mechanical Partial Zona Dissection

AH following mechanical opening of the ZP and showing an increased implantation rate was first reported in 1990 [11]. The method of partial zona dissection (PZD) used was similar to that described for oocytes, to assist oocyte zona pellucida penetration by spermatozoa [23] with no preincubation of the embryos in sucrose.

Embryos denuded of corona cells are micromanipulated in microdrops of HEPES-buffered medium under paraffin oil. The procedure is performed at 37°C, under an inverted microscope. The embryo is held with a holding pipette, and the ZP is tangentially pierced with a microneedle from the 1 to the 11 o'clock position. The embryo is released from the holding pipette, and the part of the ZP between the two points is rubbed against the holding pipette until a slit is made in the ZP. The embryo is washed twice in fresh culture medium and placed in the transfer dish. Three-dimensional-PZD (3D-PZD) in the shape of a cross has also been described [24]. The procedure starts as conventional PZD, and a second cut is made in the ZP under the first slit. A cross-shaped cut can be seen on the surface of the ZP. This method allows the creation of larger openings while permitting protection of the embryo by the ZP flaps during embryo transfer. A new technique called "controlled zona dissection" (CDZ) has been described as a variation of PZD [25]. The embryo is held at 8 o'clock position by a beveled opened holding pipette and a thin angled hatching needle with a blunted tip pierce the ZP at 5 o'clock position. The hatching needle is inserted deeply into the holding pipette until the embryo is pushed to the angle of the hatching needle. The curve of the needle is then pressed against the bottom of the dish to cut the pierced ZP. A large slit (two thirds of embryo's diameter) created by CDZ enhances significantly the rate of complete in vitro hatching of blastocysts compared to 3D-PZD.

Chemical Zona Drilling

The human ZP can be dissolved in a low-pH solution as the acid Tyrode's solution (AT solution: pH of 2.2–2.6). AT solution can be prepared in the laboratory with the protocol of Hogan et al. [26] and adjusted to a pH of 2.5 or can be purchased commercially.

Selective AH to promote blastocyst hatching involving chemical opening of the zona with AT was first reported in 1992 [27]. One advantage of AT drilling compared with PZD is the possibility of increasing the size of the hole in the ZP. Large holes have proved to be more efficient for enhancing hatching and avoiding embryo entrapment. In order to perform chemical zona drilling, the embryo is held with a holding pipette in such a way that the micropipette containing AT (internal diameter 3–5 mm) and located at the 3 o'clock position faces a large perivitelline space or an area with cytoplasmic fragments of the embryo. The acid solution is gently delivered with the help of a microinjector over a small area of the ZP, with the tip of the pipette positioned very close to the zona. Accumulation of AT in a single area must be avoided. As soon as a hole in the ZP is created, suction is applied to avoid excess AT entering the perivitelline space. If the inner region of the ZP is difficult to breach, creation of the hole can be facilitated by pushing the AT micropipette against the ZP. Extracellular fragments can also be removed by aspiration during the procedure [27]. It is necessary to rinse the embryo several times in fresh culture medium immediately after the AT hatching procedure, prior to returning it back into the culture dish, to avoid detrimental effects of the acid solution on the blastomeres.

The ZP can also be chemically removed from embryos reaching the blastocyst stage on day 5 of in vitro culture. Embryos are placed into a 30 mL drop of prewarmed AT solution under mineral oil for approximately 10 s, followed by careful washing in five drops fresh culture medium.

Enzymatic Zona Digestion

Blastocyst transfer after enzymatic treatment with pronase of the ZP, to either soften or remove totally the zona before transfer, has shown high pregnancy and implantation rates in patients with previous repeated implantation failure [21]. The first delivery of a healthy baby after an enzymatically treated zona-free blastocyst transfer was reported on 1997 [28].

The ZP can be softened or totally dissolved after treatment with pronase at concentrations of 10 IU/mL in medium under oil in a 5–6% CO_2 in air atmosphere and at 37°C for exposure periods of no longer than 1.5 min. Embryos have to be washed several times in fresh medium after the AH procedure. There are some evidence that pronase treatment in blastocysts for longer periods may cause adverse effects on the trophectoderm and inner cell mass [29].

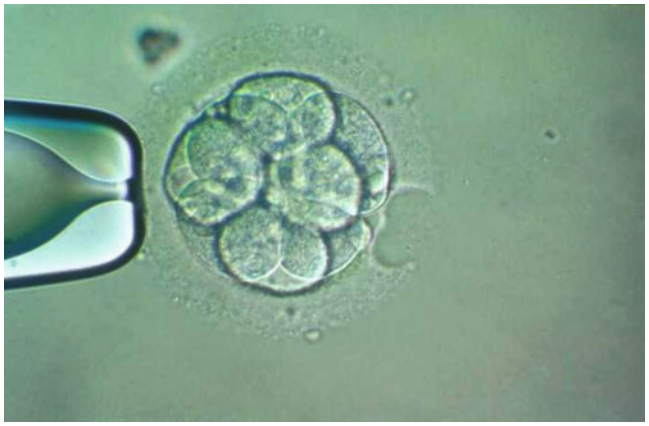

Fig. 49.1 Laser-assisted hatching. The zona pellucida (ZP) of an eight-cell embryo drilled through photoablation by laser technology: a single opening of ~30 mm is created by dissecting the full thickness of the ZP. (From Veiga et al. [18], with permission)

Laser Technologies

The application of a laser on the ZP for AH results in photoablation of the zona pellucida. For fast and efficient clinical use of laser systems in AH, it is important that the laser is accurately controlled and produces precise ZP openings without thermal or mutagenic effects [18].

The first use of a laser for ZP drilling was reported in 1991 with an ArF excimer laser (ultraviolet (UV) region, 193 nm wavelength) [30]. This laser system is a contact mode laser and makes necessary to touch the ZP with the laser-delivering pipette. Another contact mode laser, the erbium:yttrium–aluminum–garnet (Er:YAG) laser (2,940 nm radiation), has also been used for ZP drilling and thinning, and its safety and efficacy have been demonstrated in clinical practice [31]. No degenerative alterations on the ZP and membrane of embryos were observed using light and scanning electron microscopy after ZP drilling with such a system [32]. But the necessity of sterile micropipettes and optical fibers to deliver the laser beam to the target is the main disadvantage of contact mode lasers.

Noncontact laser systems allow microscope objective-delivered accessibility of laser light to the target. Laser propagation is made through water, and as it avoids the UV absorption peak of DNA, no mutagenic effect on the oocyte or embryo is expected [18]. Several noncontact laser systems have proved to be useful for ZP drilling in the mouse model [33, 34]. Rink et al. introduced an InGaAsP noncontact diode laser that use infrared light at 1.48 mm wavelength [35]. Its high absorption in water causes rapid and precisely localized heating of the target region. The drilling mechanism is explained by a thermal effect induced at the focal point by absorption of the laser energy by water and/or ZP macromolecules, leading to thermolysis of the ZP. Laser absorption by the culture dish and medium is minimal. The effect on the ZP is greatly localized, and the holes obtained are cylindrical and precise. Exposure time (1–20 ms) can be minimized. The system is compact and easily adapted to all kinds of microscopes. The size of the hole is related to the laser exposure time. The safety and usefulness of the system has been demonstrated in mice and human [36, 37].

This is actually the laser system preferred to drill the ZP of human embryos since it is simple, quick, and easy to use.

AH may be performed a few hours or immediately prior to ET. A culture dish with several 10 mL drops of HEPES-buffered culture medium and overlaid with prewarmed mineral oil has to be prepared shortly before the procedure. Embryos selected for transfer are placed individually in separate drops, and the dish is placed on the heated stage of the inverted microscope equipped with the laser technology. It is recommended to stabilize the embryo with a holding micropipette. An area between two blastomeres with a large perivitelline space has to be chosen for laser drilling.

Routinely, a single opening of ~30 mm (about 10% of the ZP circumference) is created by dissecting the full thickness of the ZP (Fig. 49.1). Thinning the ZP can be done focusing the laser shoots to the outer layer of the zona. Several pulses (2–8 pulses) are necessary to complete the cut through the ZP or to thin a quarter of the embryo circumference.

It has to be taken in account that diode laser beam produces superheated water approaching 200°C on the beam axis. The action of the laser must be strictly limited to the targeted region of the ZP, since focused laser irradiation on a specific cell would cause damage and would probably be lethal to that cell. Following irradiation, the heat is conducted away from the target and is dissipated into the surrounding medium. The potential to damage blastomeres adjacent to the hole created by the laser is minimized by using short pulse durations (≤5 ms)and mild laser power (~100 mW) at a safe distance from the blastomeres [38–40].

At the end of the procedure, the embryo is immediately washed, replaced in the culture media, and returned to the incubator.

Embryo Stage to Perform Assisted Hatching

AH can be performed at different stages of embryo development, but the first results published were obtained after performing AH to early stage developing embryos, [11, 27] probably because embryo transfer was routinely performed on day 2 or 3 of culture.

Early Embryo Stage

At day 3 of development, normal embryos should have 6–8 noncompacted blastomeres. AH, thought to be the best method currently available to overcome the ZP barrier in unexplained implantation failure in fresh embryo transfer, is usually performed on day 3. AH prior to transfer, by total disruption or by thinning of the ZP, seems to enhance pregnancy and implantation rates in some selected groups of patients.

Fragment Removal

Embryo development is sometimes associated with a certain degree of cytoplasmic fragmentation. The potential of fragmented embryos for implantation is determined partly by the distribution of fragments. The use of microsurgical fragment removal may alter the course of development for some embryos and can improve their implantation potential [41].

Embryo fragmentation can be reduced by mechanical aspiration after AH prior to embryo replacement. Embryo denuded of corona cells is micromanipulated in microdrops of HEPES-buffered medium under paraffin oil. As mentioned above, the procedure is performed at 37°C, under an inverted microscope with micromanipulation system. The embryos stabilized with a holding pipette held at the 9 o'clock position. AH can be performed mechanically, chemically, or with laser. A 10-mm micropipette is oriented at the 3 o'clock position adjacent to the area with cytoplasmic fragments of the embryo where the hole has been created. Cytoplasmic fragments are removed by gentle aspiration. The embryo is then rinsed several times and returned to the standard culture media to the incubator until transfer.

Blastocyst Stage

Although AH is usually performed on early cleavage stage embryos, it seems to be more physiologically feasible to assist this process in vitro at the blastocyst stage as in vivo hatching occurs at that stage just prior to implantation.

AH performed at the blastocyst stage seems to enhance embryo implantation [21, 22]. It is preferable to apply AH to day 5 blastocysts (initial blastocysts) than to expanded blastocysts to avoid harmful effects on trophectoderm cells.

As mentioned above, to perform partial zona drilling or zona thinning, the procedure is performed in microdrops of HEPES-buffered medium under paraffin oil, at 37°C, under an inverted microscope with micromanipulation system. The blastocyst is stabilized with a holding pipette held at the 9 o'clock position. AH can be performed to the blastocyst mechanically, chemically, or with laser. The embryo is then rinsed several times and returned to the incubator until transfer.

Full ZP removal has shown to improve the outcome of blastocyst transfer after vitrification [22]. It has been observed that human blastocysts completely hatched in vitro do not stick to the surface of the culture dishes or the inner surface of embryo replacement catheter. Therefore, these blastocysts can be handled like intact blastocysts [18]. Total ZP removal of blastocyst can be done enzymatically or chemically as described above.

Frozen–Thawed Embryos

Elasticity and thinning of the ZP can be adversely influenced by the freezing–thawing process. The stress induced by the freeze–thaw process may lead to zona hardening, impairing the ability of blastocysts to hatch. AH in frozen–thawed cycles has been shown to increase pregnancy and implantation rates both in early cleavage stage embryos [42, 43] and blastocysts [22].

Frozen–vitrified–thawed early stage developed embryos or blastocysts can undergo AH by the different methods described above.

Necrotic Material Removal

The implantation rate of embryos partially surviving cryopreservation seems to be impaired. It can be speculated that the lysed blastomeres are producing factors as they degrade that are detrimental or toxic to the other cells in the embryo or that may disrupt cell-to-cell communication. There is evidence of possible benefits of the removal of lysed cells from frozen–thawed embryos [44, 45] with no clear explanation about such benefits.

Day 2 or day 3 frozen embryos are thawed 1 day before embryo transfer. AH of frozen–thawed embryos is usually performed shortly before embryo transfer. However, removal of lysed material is easier when AH is performed soon after the embryo thawing process. The frozen–thawed embryo, placed in a dish with microdrops of HEPES-buffered medium

under mineral oil, at 37°C, under an inverted microscope with micromanipulation system, is stabilized with a holding pipette held at the 9 o'clock position. AH is performed as usually by creating an opening in the zona pellucida using AT or laser system. The AH micropipette (10 mm) has to be oriented to the necrotic material area of the embryo where the hole has been created. Lysed blastomeres are removed by gentle aspiration. Embryo is then washed several times in fresh medium and returned to the incubator for further culture till transfer.

Potential Adverse Effects

Patients whose embryos are hatched are often treated with antibiotics and steroids before and after embryo transfer, exposing them to the potential risks and side effects of such treatments.

Embryo Damage

The AH procedure may be associated with specific complications independent of the IVF procedure itself, including lethal damage to the embryo and damage to individual blastomeres with reduction of embryo viability, probably as an adverse effect of embryo micromanipulation.

Risk of Increased Monozygotic Twinning

Artificial manipulation of the ZP has been associated with an increased risk of monozygotic twinning [46, 47]. Artificially induced damage to the ZP may be related with inner cell mass splitting during hatching and thus may result in monozygotic twinning. The size of the hole performed by ZP micromanipulation could be a conditional factor. However, a recent systematic review found insufficient data to assess the impact of AH on monozygotic twinning [48].

Commentary

It is questionable whether different methods of AH yield similar outcomes. Mechanical hatching by PZD is limited by the difficulty of creating a hole of consistent size. The use of AT for zona drilling can carry potential problems due to its variability and possible embryotoxicity. Enzymatic methods to dissolve or thin the zona seem to be effective and safe. Although the equipment may be expensive, the use of a 1.48-mm diode infrared noncontact laser system for zona drilling appears to be the most suitable method for AH in the IVF laboratory: it offers a low potential risk and is quick and relatively simple to perform with high consistency between operators.

Key Issues

Higher clinical pregnancy and implantation rates have been reported after AH, but a recently published Cochrane meta-analysis concluded that, although live birth should be considered the primary outcome, there is insufficient evidence to determine any effect of AH on live birth rates [48]. Thereby, the conclusions concerning AH benefits and taking in account the variability in methods and studies performed are as follows:

1. AH does not enhance the outcome in patients undergoing their first IVF attempt.
2. AH increases the pregnancy rate in patients with previous implantation failures or poor prognosis in both fresh and frozen embryo transfer.
3. It is not clear whether AH is beneficial for patients of advanced age, embryos with a thick ZP, or for frozen–thawed embryos.
4. More robust trials with adequate methodological quality and power to investigate the role of assisted hatching in different groups with live birth reports and multiple pregnancy data are needed.
5. Currently, there is insufficient evidence to recommend assisted hatching as a routine technique in patients undergoing ART.

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Part IX

Biopsy Procedures on Oocytes and Embryos

Polar Body Biopsy

50

Markus Montag, Maria Köster, K. van der Ven, and Hans van der Ven

Abstract

Biopsy of the first and second polar bodies allows investigation of the chromosomal and genetic constitution of the corresponding oocyte. Opening of the zona pellucida prior biopsy can be performed using a bevelled pipette, by 3D partial zona dissection or by a non-contact diode laser. Acid tyrode solution is not suitable for zona drilling in oocytes. The biopsy procedure through such an opening allows for the simultaneous or sequential removal of the first and/or second polar body. Once biopsied, polar bodies can be analysed by fluorescence in situ hybridization or by array-comparative genomic hybridization for numerical and structural chromosomal aberrations or by polymerase chain reaction-based techniques for monogenetic diseases. Biopsy of the first and second polar bodies has no impact on further embryo development.

Keywords

Preimplantation genetic screening • Polar body • Biopsy • Oocyte • Laser • Fluorescence in situ hybridization

Polar body biopsy was first proposed in 1990 by Verlinsky and collaborators [1]. Since then, several groups have applied polar body biopsy to a variety of diagnostic applications like 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. Therefore, removal of both polar bodies for the purposes of genetic diagnosis should have no deleterious effect on the developing embryo.

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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.

Timing

An oocyte presenting a first polar body is usually considered to be in metaphase II. However, recent investigations using

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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 [12]. 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 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.

In view of this, removal of the first and second polar bodies can be done at separate time points or at the same time point. Simultaneous biopsy of the first and second polar bodies is best accomplished in a time window of 6–14 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 which 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]. Simultaneous biopsy requires only one manipulation and helps to reduce stress to the oocyte.

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.

Chemical Opening

Acidic tyrode solution was the first method ever used for opening of the zona pellucida by chemical means [13]. 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 [14]. Therefore, since both the oocyte and polar body are sensitive to the effects of acid, zona drilling by acidic tyrode solution is unsuitable for polar body biopsy.

Mechanical Opening

A very efficient mechanical technique was elaborated by Cieslak et al. [15] and is based on 3D 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 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 will assist 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 towards 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.

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 [16] has entered the field of polar body [11, 17] and embryo biopsy [18] and has helped in reducing the rate of biopsy damages as well as the time required [19].

Biopsy Procedure

The whole procedure of laser-assisted polar body biopsy is illustrated in Fig. 50.1.

Laser-assisted polar body biopsy is best accomplished when the oocyte is affixed 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 towards the holding capillary, and 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 18–25 μm, 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 which cannot be reliably diagnosed as a result of technical problems during the biopsy procedure [20]. Another benefit is that laser drilling and subsequent biopsy can be performed without changing the culture dish or the capillaries in contrast to zona drilling using

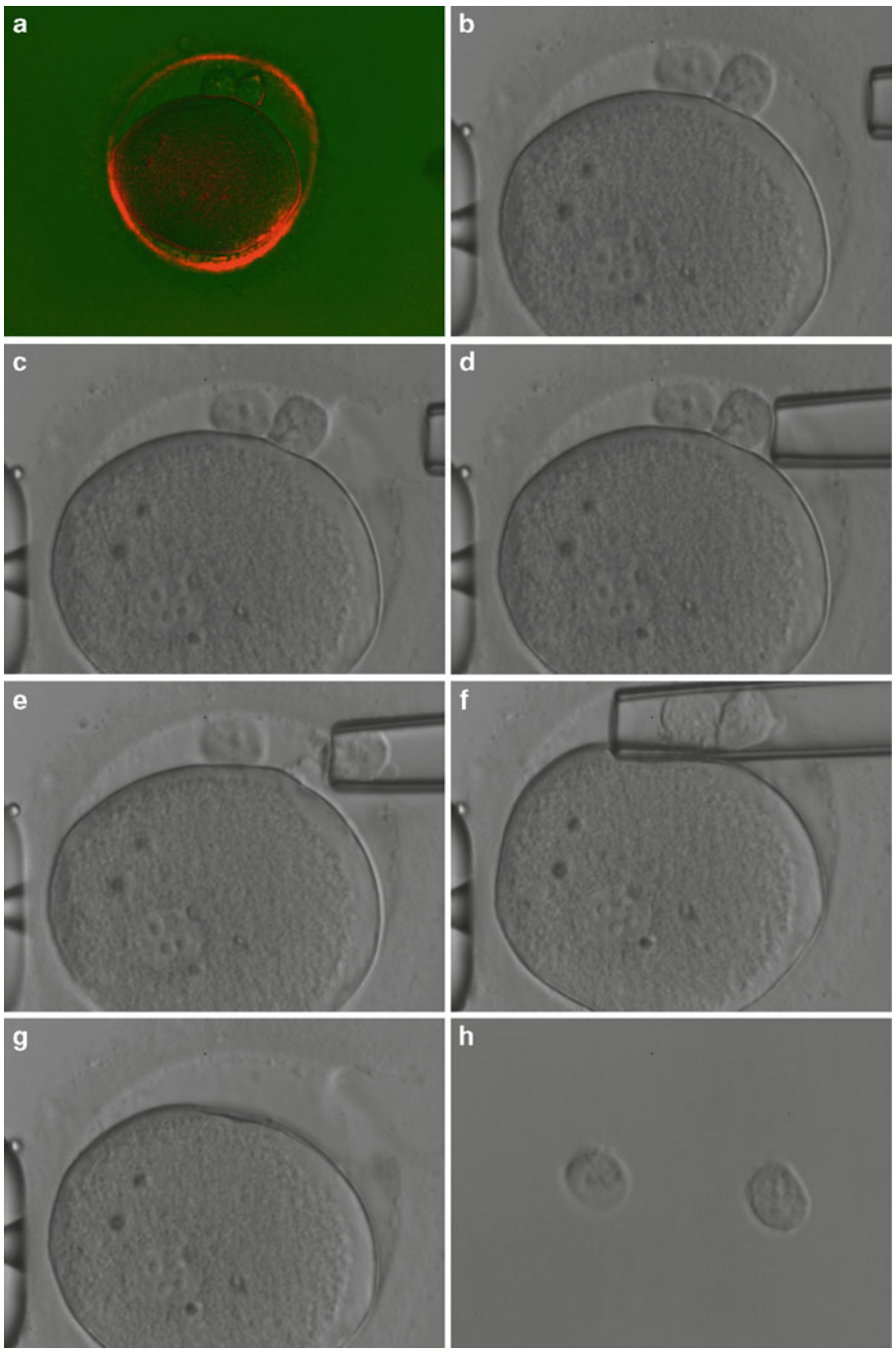

Fig. 50.1 Polar body biopsy following polarization microscopy. Prior to biopsy of the first polar body, the presence of a connective spindle bridge between one of the polar bodies and the oocytes was excluded by polarization microscopy (**a**). For biopsy, the first and second polar bodies were aligned with a holding capillary so that the second PB faced to the biopsy capillary (**b**). Using a non-contact 1.48-µm diode laser, an opening was introduced into the zona pellucida using 2–3 laser shots (**c**) through which the biopsy capillary could be easily introduced (**d**). The second polar body is usually connected to the oolemma via a cytoplasmic strand (**e**). 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 (**e**). Instead the capillary is pushed slowly over the second PB and towards the first PB (**f**). Once the first PB enters the capillary, the strand between the second PB and the oolemma will break due to shear stress, and both polar bodies can be easily removed (**f**) leaving the oocyte without any damage (**g**). Polar bodies should be placed in one droplet for further processing for FISH analysis (**h**) or in two different droplets if a PCR-based analysis will be performed

acidic tyrode solution. This may help to prevent contamination of samples to be diagnosed by sensitive techniques such as polymerase chain reaction (PCR).

Pitfalls

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 the laser seems to be easy and straightforward, it is still essential that the technique is trained properly in order to avoid possible pitfalls [21]. Laser opening of the zona can be done at very high precision and giving reproducible results. Consequently, it was shown that laser-assisted biopsy does not interfere with further development of mouse embryos [17] as long as the laser is used in a proper way [21] and a few examples will be given below.

Laser-drilled openings will 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.

Dependent on the size and position of laser openings, inappropriate hatching may occur at the blastocyst stage [21]. If the biopsy of both polar bodies is done 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 [21].

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 degeneration [21].

Independent of the method used for biopsy, it is extremely important to note the shape of the polar body. Especially a fragmented polar body must 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.

Isolation of Polar Bodies

Once polar bodies are biopsied, they need to be transferred for further analysis by FISH or PCR. This transfer is a crucial step as it bears the risk of loss of material or even of contamination.

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 polar bodies, a special pretreatment like hypo-osmotic swelling or proteinase/pronase treatment prior 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 will attach to the slide within a small area and that the fluid will dry 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 will prevent floating and rupture of the polar bodies. The drying process must 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 [22].

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 array-CGH. Therefore, the first and second polar bodies are released after biopsy in different droplets with medium in a dish covered with mineral oil. These droplets should be rather large (approx. 10 μL) as this will facilitate to aspirate the polar bodies without sucking up some mineral oil. For PCR, the polar bodies need to be transferred into a PCR tube. This can be easily done by preloading the PCR tube with 1.6 μL buffer (PBS or cell extraction buffer). Using a low-volume pipette (0.2–2.0 μL), one polar body is aspirated in a total of 0.4 μL medium and released into the buffer in the PCR tube by pipetting several times up and down. This process must be done in a clean environment, preferably a lamina flow bench, in order to avoid any contamination with other cells or genetic material.

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 methods are mechanical by 3D zona dissections or by laser. 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 previous studies on the success of polar body diagnosis for PGS. Proper training as well as future improvements and refinements may help to optimize polar body biopsy and subsequent diagnosis in the daily laboratory work. Although FISH and PGS were and probably still are the predominant applications of polar body-based diagnosis, recent advances in array-CGH will change the field of polar body biopsy and boost PCR-based diagnosis [23].

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Cleavage-Stage Embryo Biopsy

51

Alan R. Thornhill

Abstract

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. 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. 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. 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 removal of one or more cells. Many of the biopsy techniques currently in use for human embryos were pioneered in animal models, notably the mouse. 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. This chapter will focus on cleavage-stage embryo biopsy since the majority of PGD centers and clinical cases reported have employed this technique.

Keywords

Preimplantation genetic diagnosis • Cleavage-stage embryo biopsy • Whole genome amplification • Zona pellucida penetration • Blastomere removal

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 51.1). 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

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Table 51.1 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 day of biopsy
		Possible selected cell allocation to TE/ICM

Table 51.2 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 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?	

micromanipulation process involving the penetration or removal of part of the zona pellucida surrounding the oocyte or embryo followed by 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 51.2). This chapter focuses on cleavage-stage embryo biopsy since the majority of PGD centers and clinical cases reported have employed this technique [3].

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).

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 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 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, 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].

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 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-morpholinepropanesulfonic 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 aspirated from the biopsy drop.

Non-contact 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 [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 ease of use and the 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. 51.1). 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 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

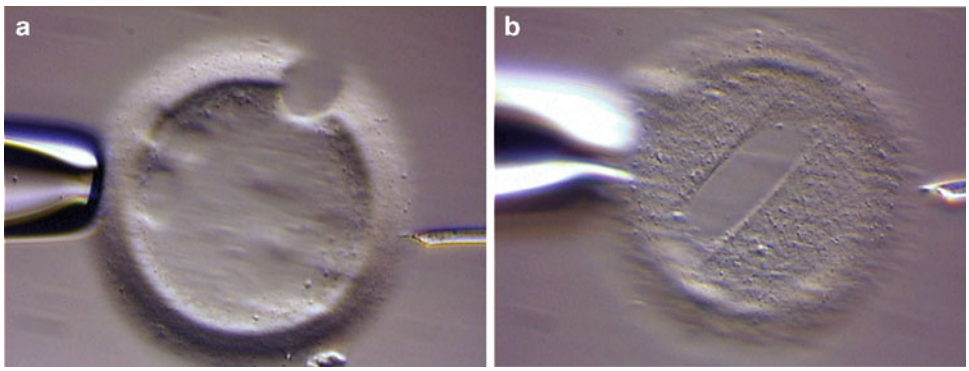

Fig. 51.1 Empty human zona pellucida after thermal ablation using noncontact laser. (**a**) Cross-sectional view as used during biopsy procedure indicating aperture through which biopsy aspiration pipette is passed. (**b**) The same zona pellucida rotated through 90° to show the path of the laser

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) 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.

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.

Aspiration

If performing aspiration in conjunction with zona drilling using acidified Tyrode's, 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.

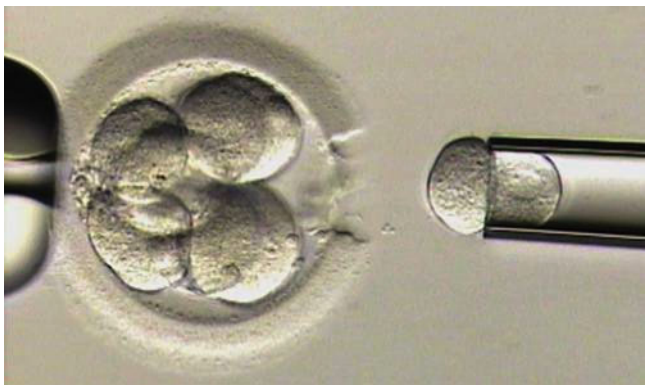

Fig. 51.2 Human cleavage-stage embryo from which a single blastomere with a single visible interphase nucleus is being removed by micromanipulation after laser ablation of the zona pellucida

A typical procedure for cleavage-stage biopsy using laser and blastomere aspiration is illustrated in Fig. 51.2. 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 culture. The blastomere should be washed extensively in handling medium before proceeding to the analysis.

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) 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.

Practical Considerations for Embryo Biopsy

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.

Timing of Biopsy

Most 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.

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 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 removal of two blastomeres decreases the likelihood of blastocyst formation, compared with 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 one 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].

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 many fold 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 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 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.

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. 51.2). 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.

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.

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.

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 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.

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Embryo Biopsy for PGD: Current Perspective

52

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Abstract

To date, 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.

Keywords

Embryo biopsy • Blastocyst-stage biopsy • Preimplantation genetic testing • Aneuploidy risk • Translocation testing

This chapter sets out to examine the major recent advances in embryo biopsy, specifically blastocyst-stage biopsy and preimplantation genetic testing and diagnosis (together *PGD*) which are transforming singleton live birth rates in families at risk of passing on monogenic diseases or chromosomal translocations.

The Development of Embryo Biopsy

Embryo biopsies for clinical PGD generally were performed at day 3, when the embryo typically was at the 6- to 8-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 and second polar bodies—either sequentially on day 0 and day 1, or both at 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

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Table 52.1 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 52.2 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%, $P < 0.01$)

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 3–5, 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 52.1) 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 52.1 shows the technical outcome data for the embryos biopsied (595 for day 3; 656 for days 5–6), with an 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 52.2 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.

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.

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 the 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 revealing 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.

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 52.1 and 52.2) 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 the 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 5-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}5-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 52.3 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 52.3 Pregnancy rates after preimplantation genetic screening for aneuploidy from biopsy of blastocysts on day 5 or 6 of development using 5- or 7-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%	1,194	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%), which while 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.

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* (produce 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 52.4 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 52.2), possibly

Table 52.4 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

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, 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 3 pregnancies among 7 couples with day 3 biopsies, each of which went to term, and 8 pregnancies among 7 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%.

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.

The Near Future for Translocation Testing

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 52.4 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.

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.

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 usually interphase 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 number of fluorophore colors falls far short of the minimum of 24 required. 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 2 or 3 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 1 or 2 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 requires 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.

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 two sex chromosomes to a resolution of ten 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 reported 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 for Human Reproduction. 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.

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Microarrays and CGH for PGD of Chromosome Abnormalities and Gene Defects

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Gary Harton and Santiago Munné

Abstract

Preimplantation genetic diagnosis for aneuploidy has become a fairly routine part of assisted reproductive technology worldwide. While the theory of aneuploidy screening makes scientific sense, no randomized controlled trial (RCT) using fluorescence in situ hybridization (FISH) has shown a benefit of screening embryos for common chromosome anomalies, although the reasons for this failure may be center-related. Here we discuss both the positive and negative aspects of FISH technology and review the literature to this point. Topics such as mosaicism of early embryos and the limitations of FISH technology, as well as poor technique in the laboratories carrying out the embryology and genetic diagnostics, are discussed. New array-based testing platforms that allow for testing of all 24 chromosomes have shown promise in a few recent publications and abstracts at scientific meetings. The two principal array-based platforms are explained, then compared and contrasted, followed by a discussion of validation strategies for new technologies. Finally, the current state of the clinical use of array-based testing is reviewed. At least one RCT using arrays has already shown the benefit of aneuploidy screening.

Keywords

Preimplantation genetic diagnosis • PGD • PGS • Array CGH • CGH • Aneuploidy • Embryos

Fish and Array-Based Testing

More than 50% of cleavage-stage embryos produced in vitro are chromosomally abnormal, increasing to up to 80% in women over 42 years of age [1–4]. Although some abnormal embryos arrest during extended culture, most do not, and even at the blastocyst stage, more than half of all embryos are abnormal (mean maternal age 38 years) [5]. The majority of numerical chromosome abnormalities detected in embryos are not compatible with implantation or birth which negatively affects the success of assisted reproductive treatments.

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The detrimental effect of aneuploidy is illustrated by the high prevalence of chromosome abnormalities detected in spontaneous abortions, exceeding 70% in some studies [6–11]. It has been hypothesized that selection of embryos for transfer based on chromosome normalcy (euploidy) could improve success rates in assisted reproductive procedures [12]. This process is known as preimplantation genetic diagnosis (PGD) of aneuploidy or preimplantation genetic screening (PGS). For a glossary of terms (see Glossary).

Shortcomings of Pre-Array Technologies

The first PGD strategies to be described employed fluorescence in situ hybridization (FISH) analysis of cells biopsied from day-3 embryos [12–16], trophectoderm cells biopsied from blastocyst-stage embryos [17], or polar

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bodies biopsied from oocytes or zygotes [18–21]. The FISH methods allowed analysis of 5–12 chromosomes in each oocyte or embryo but were unable to provide a full evaluation of the chromosome complement. Nevertheless, that was enough to detect more than 80% of chromosomally abnormal embryos detected by array technology [22].

Some studies utilizing FISH-based strategies reported an improvement in implantation rates, reduction in spontaneous abortions, and/or an increase in take-home-baby rates [15, 21, 23–32]. However, these studies were not randomized. Other studies, some performed in a randomized fashion, did not produce significant improvements or showed a detrimental effect of PGD for aneuploidy [33–36]. Several reasons for these conflicting results have been advanced.

The biological argument, which does not explain differences in reports, but attempts to understand why some studies observed a negative effect on outcome, argues that cleavage-stage embryos have such high rates of chromosomal mosaicism that any analysis based upon a single cell is unreliable. Although it is true that mosaicism is common in cleavage-stage embryos (about 30% according to FISH analyses) [1–4], the majority of these embryos display chromosome abnormalities in every cell. In such cases, the biopsied cell may not be chromosomally identical to the remaining cells of the embryo, they may contain errors affecting different chromosomes, but the clinical diagnosis of "abnormal" is still valid. Large follow-up studies of preimplantation embryos diagnosed using FISH estimate only a 5–7% error caused by mosaicism [15, 37] when embryos are reanalyzed in all their cells by FISH. Even less, only 2% when analyzed by array CGH [38], thus mosaicism is unlikely to be the primary cause of poor outcomes following PGD.

The most probable cause of intercenter differences in PGD results are variations in the biopsy and genetic technologies employed. These encompass all aspects of the process and have been previously reviewed [39]. Here we will discuss briefly only a few of the key factors. Probably the most important variable in PGD is the embryo biopsy itself. One of the studies showing no difference in IVF outcome following PGD involved biopsy of two cells from each cleavage-stage embryo [33]. However, the same group later reported that two-cell biopsy, in contrast to single-cell biopsy, is detrimental to embryo development [40].

Even biopsying one cell in suboptimal conditions could be extremely damaging to embryo potential. A study conducted by Mastenbroek et al. [35] reported an astonishingly high rate of diagnostic failure (20%), resulting in many embryos being transferred without a diagnosis. The implantation rate of these undiagnosed embryos was 59% lower than the control. In this case, the only difference between the control and test groups appears to have been the biopsy, suggesting that embryo viability was drastically reduced by the biopsy procedures used in the clinics involved.

The second most important factor in obtaining good results can be summarized in the "error rate." The steps after biopsy involve fixation, FISH with a variety of potentially different protocols and probes, and cell scoring. However, the overall accuracy of these steps can be summarized in a single number, which is the error rate of a PGD laboratory. This error rate can be obtained by reanalyzing all the cells of nonreplaced embryos (abnormal embryos and arrested normal embryos) and determining if the original diagnosis was correct. Unfortunately, error rates vary widely, ranging from 2–7% [2, 15, 38] to 40–50% [22, 41] depending on the PGD laboratory. As shown in a recent review, error rates around 50% will in fact decrease implantation rates [39].

When performed using appropriate, well-validated methods, FISH can detect 90% of the chromosome abnormalities detected by CGH [16, 42], and some PGD laboratories do appear to obtain consistently good results with FISH and cleavage-stage embryo biopsy. Regardless, the field of PGD is evolving away from biopsying at this stage of embryo development and is increasingly focusing on biopsy of polar bodies from oocytes or zygotes or removal of trophectoderm cells from blastocysts. These embryonic stages may be more resilient to technical manipulation. Additionally, the limited chromosomal screening conveyed by FISH is increasingly being replaced by comprehensive methods of DNA analysis, which detect close to 100% of chromosomal abnormalities. The new wave of aneuploidy testing technologies is extremely redundant (each chromosome tested multiple times at different sites), readily automated, less subjective, and theoretically less prone to errors.

Comprehensive DNA Analysis Techniques

Here we will cover three techniques that are currently being used for PGD of chromosome abnormalities. Comparative genome hybridization (CGH) was first applied to day-3 embryo biopsies [43–47]. However, CGH is time consuming and is incompatible with day-3 biopsy and transfer by day 5, necessitating cryopreservation of embryos while testing is carried out. At the time that it was first applied, embryo freezing was a relatively inefficient technique and the low survival rate of thawed embryos likely neutralized any beneficial effects of CGH. For these reasons, CGH was temporarily abandoned and not applied again until the development of vitrification [48]. In conjunction with vitrification, CGH has been clinically applied to polar bodies [49–51] and blastocyst biopsies [5, 52]. The combination of CGH, blastocyst biopsy, and vitrification significantly improved implantation rates in a recent study, from 46.5% in controls to 72.2% in cycles with screening, with nearly 100% of blastocysts surviving biopsy [5]. However, many clinics are not yet proficient at blastocyst culture and vitrification. Furthermore, freezing

adds extra cost to the cycle, and a majority of patients prefer to have a fresh cycle. Thus, for the time being, day-3 biopsy combined with comprehensive chromosome analysis remains the choice for most physicians and patients.

Two other techniques, microarray CGH (array CGH or aCGH) [53–57] and single-nucleotide polymorphism (SNP) microarrays [58–60], can be used for comprehensive chromosome analysis of single cells from day-3 biopsy and yield results in 24 h. The rapid turnaround time for these methods eliminates the need to cryopreserve embryos while testing is carried out.

Array CGH (aCGH) is already widely used for the cytogenetic analysis of prenatal and postnatal samples [61–66] since it is rapid and cost-effective and allows chromosomal regions to be screened at high resolution. Several types of aCGH platform 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 generally recommended since at that level, the difference between clinically significant duplications/deletions and normally occurring copy number variations is less clear. A microarray recently validated for PGD had 4,000 probes and thus covered ~25% of the genome sequence [58]. Microarray CGH has a similar accuracy rate to conventional CGH and should therefore be capable of producing similar results to those obtained in the promising CGH study performed by Schoolcraft et al. [5].

CGH and aCGH provide 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 many distinct loci, the diagnosis is very accurate.

Chromosome imbalances (aneuploidies, unbalanced translocations, deletions, and duplications) are easily detected using CGH and 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? In a recently submitted paper by Munne et al. (personal communication), about 7.7% ($n=91,073$) of the supposedly 2PN embryos tested were polyploid or haploid but the majority of them had additional abnormalities detectable by CGH or array CGH and only 1.8% of all embryos were homogeneously polyploid or haploid. Furthermore, of those, 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 or CGH.

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 [58, 60, 67].

All of the new generation of chromosome screening methods (CGH, aCGH, and SNP microarrays) rely on whole genome amplification (WGA) to amplify DNA from the single cell or small number of cells removed from a developing embryo [68]. 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. Currently, 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.

Currently, a few PGD groups around the world are validating SNP microarrays and analysis software for clinical use in PGD for aneuploidy screening. It is expected that data from the clinical use of SNP microarrays will closely match the data from CGH and aCGH testing. While the technologies differ greatly, both types of arrays (CGH-based and SNP-based) are trying to answer the same 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 cleaning up of data from SNP microarrays have been developed: qualitative methods, looking only at the inheritance of specific SNPs and requiring

comparison with parental DNA samples; quantitative approaches, assessing only the intensity of SNP calls; and 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 [58]. 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. This can misdiagnose a substantial amount of embryos since 30% of aneuploid embryos contain mitotic abnormalities (mosaics) [15]. 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 [60, 67]. All of the analysis 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 offer some advantages over aCGH: (a) if qualitative analysis is employed, SNP-based microarrays can also detect the parental origin of any chromosome 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 error where the paternal chromosome was randomly recruited as the extra chromosome. 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%) [69], 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. However, if a laboratory is using aCGH, a similar test can be performed by utilizing a small aliquot of the DNA produced by WGA to perform conventional DNA fingerprinting; (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 precycle parental testing that was ultimately unnecessary.

Validation of aCGH and SNP Arrays

Due to the intrinsic and often unforeseen problems with every new technology, a novel method should always be validated against other, more established methods [70]. Assessing a new approach against itself may preclude the detection of technique-related flaws. Thus, validation by inadequate methods such as the analysis of cell lines with defined chromosome abnormalities which cannot mimic mosaicism and other peculiarities of the cell being tested; analysis of eggs or embryos by one technique with analysis of polar bodies or the remainder of the embryo by the same technique which will preclude identifying abnormalities not detectable by that technique; blindly replacing undiagnosed embryos (either by single embryo transfer or fingerprinting the embryo) and following pregnancies and clinical losses to determine the fate of each tested embryo which does not account for the status of nonimplanted embryos; or using the SNP calls in one chromosome as internal controls for other SNPs in that same chromosome [60] may lead to false assumptions. 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 a new technique is to reanalyze those embryos that were not transferred to the patient, either because they underwent arrest or because they were diagnosed chromosomally abnormal. The reanalysis of these embryos should be done with another well-established technique, the "gold standard." This would discern shortcomings of the new method under evaluation and account for issues related to embryo biology, such as mosaicism. The only problem with this approach is that euploid arresting embryos may become abnormal (karyokinesis without cytokinesis) from day 3 to day 5 before reanalysis [71, 72] and there is a scarcity of nonreplaced normal embryos.

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 [67, 73], SNP microarray reanalysis of embryos previously analyzed by SNP arrays [59], 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). In a recent study, 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 [58]. This is even lower than the 7% error rate expected solely from mosaicism as calculated in FISH studies [15]. Most likely, analyzing all of the chromosomes leads to the ascertainment of more chaotic embryos, further lowering the overall error rate.

Clinical Results

Of the techniques discussed, CGH is the one for which the greatest quantity of clinical data is available [5, 49, 50, 52]. Sher et al. [49] detected a 74% ongoing pregnancy rate per transfer and 63% per retrieval in women with an average age of 37.5 years. For patients of a similar age, receiving blastocyst transfer, Schoolcraft et al. [5] detected a significant increase in implantation rates, from 46.5 to 72.2% ($p<0.001$) following embryo selection using CGH. Interestingly, both studies showed high implantation rates and both 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 nonstimulated cycle [74]. Loss of blastocyst-stage embryos after devitrification in the study by Schoolcraft et al. was minimal (0.7%) [5].

Regarding day-3 biopsy followed by aCGH and day-5 replacement, our most recent data [75] showed that only 118/151 PGD cycles had normal embryos for transfer in a population 38 years of age. The pregnancy rate was 59% per transfer compared with 38% in controls with a transfer ($p<0.001$). The ongoing pregnancy rate for the PGD group was 54% per transfer, compared with 31.1% in controls with a transfer ($p<0.001$). These results are encouraging but not as impressive as the day-5 (blastocyst) biopsy results. It is probable that the difference between clinical results obtained using CGH and aCGH is related to the stage at which biopsy was carried out rather than to differences in the method of chromosome screening. It is very likely that aCGH will replicate the results obtained by CGH when applied in conjunction with blastocyst biopsy. In summary, although data on the clinical application of comprehensive chromosome analysis techniques is preliminary, all studies suggest a significant improvement in ART results.

Less clinical data is available from SNP-based microarrays. In presentations at ASRM, Schlenker et al. [76] reported that CGH and SNP microarrays provided the same high implantation rates after blastocyst biopsy and vitrification. Also, in an ongoing RCT using 24-chromosome analysis by qPCR, higher pregnancies rates were obtained when biopsy was performed on day 5 than in controls with no intervention [77].

Microarrays for PGD of Gene Defects

Neither CGH microarrays nor the SNP microarrays in current use can directly detect gene defect mutations. However, SNP microarrays can be used to indirectly infer the presence or absence of a chromosome segment containing a mutant gene (i.e., identification of the same SNP haplotype as the parental chromosome carrying the mutation). A diagnosis can be performed based upon this sort of information [59]; indeed, this approach has recently been applied clinically for the simultaneous detection of gene defects and chromosome abnormalities [78].

In the case of aCGH, although gene defects cannot be detected directly, enough DNA is produced during the WGA step of the procedure that an aliquot can be used for aCGH analysis of chromosome abnormalities and another taken for PCR-based analysis of gene defects.

The high levels of ADO recorded after WGA mean that direct detection of a mutation using a microarray is likely to be less reliable than existing forms of PGD. Microarray-based diagnosis will be safer using approaches such as karyomapping, where conclusions are based upon the results from multiple linked SNPs, rather than a single mutation site. While the sort of microarrays used for preconception screening are not currently suitable for PGD, it is anticipated that their use will significantly increase the identification of high-risk couples and therefore lead to an increase in the usage of genetic testing modalities such as prenatal testing and PGD.

Glossary

Allele dropout (ADO) The failure to detect an allele in a sample or the failure to amplify an allele during PCR.

Aneuploidy The condition of a cell or of an organism that has additions or deletions of a small number of whole chromosomes from the expected balanced diploid number of chromosomes.

Array comparative genomic hybridization (aCGH) A technique to detect genomic copy number variations at a high resolution level using differentially labeled DNA samples (one of unknown karyotype, one known normal karyotype) after hybridization to specific parts of the genome printed on a glass slide.

Bacterial artificial chromosome (BAC) Artificial chromosome vector derived from bacteria used for cloning relatively large DNA fragments.

Chromosome translocation A chromosomal configuration in which (usually) the ends of two nonhomologous chromosomes have become exchanged.

DNA fingerprint The derivation of unique patterns of DNA fragments obtained using a number of short repeats following polymerase chain reaction allowing discrimination between the genetic makeup of one person from another or one embryo from another.

Comparative genomic hybridization (CGH) A technique that is used to detect chromosome gain or loss by hybridizing DNA from a target cell and a normal cell that are differentially labeled with unique fluorescent dyes to a normal karyotype.

Euploidy The condition of a cell or organism that has one or more complete sets of chromosomes.

Fluorescence in situ hybridization (FISH) A cytogenetic technique that is used to detect and localize the presence or absence of specific DNA sequences on chromosomes.

Haplotype A set of closely linked genetic markers present on one chromosome that tend to be inherited together (not easily separable by recombination).

Karyotype Cytogenetic chromosome analysis with direct visualization of the chromosomes to determine chromosome number and structure/content.

Meiotic error A chromosome error arising during meiosis (reduction division) in an egg or sperm.

Microarray Sometimes called a gene chip or a DNA chip. Microarrays consist of large numbers of molecules (often, but not always, DNA) distributed in rows in a very small space. Microarrays permit scientists to study inheritance of chromosomes and gene expression by providing a snapshot of all the genes that are active in a cell at a particular time.

Mitotic error A chromosome error arising during mitosis (division) of an embryo.

Mosaicism Two or more distinct chromosomal or genetic lineages within an individual embryo.

Polyploidy A cell or an organism having three or more chromosome sets.

Preferential amplification (PA) Phenomenon that occurs during amplification from small amounts of starting DNA where one allele preferentially amplifies more than the other allele.

Preimplantation genetic diagnosis (PGD) Diagnosis of a cell from a preimplantation embryo for a specific genetic disease before embryo transfer.

Preimplantation genetic screening (PGS) Screening of a cell from a preimplantation embryo for the detection chromosomal disorders before embryo transfer.

Single nucleotide polymorphism (SNP) A variation of a single nucleotide of DNA useful in understanding and identifying a higher risk of a disease in particular people.

Whole genome amplification (WGA) The in vitro amplification of a full genome sequence, ideally with even representation of the genome in the amplified product.

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Part X

Cryopreservation

Sperm Cryopreservation

54

Fabio Firmbach Pasqualotto, Eleonora Bedin Pasqualotto, Edson Borges Jr., and Ashok Agarwal

Abstract

While the idea of freezing human male gametes has been experimented since the late 1700s, it was not until 50 years ago that human sperm were capable of being frozen and later thawed in such a way that they could fertilize an egg and initiate development. In the past several decades, the technology of cryopreservation, or maintaining life in a frozen state, has advanced considerably. With the use of modern techniques, cryopreservation of sperm to preserve an individual's ability to reproduce has become successful, safe, and widely available.

Keywords

Sperm • Cryopreservation • Male germ cell • Oxidative stress • Male infertility • Testicular stem cell transplantation • Azoospermia

The first reported observations of the effects of low temperatures on spermatozoa were performed by Lazaro Spallanzani in 1776, and the first to discuss the possible uses of sperm banks was the Italian Paolo Mantegazza, who wrote the following sentence in 1866: "It might even be that a husband who has died on a battle-field can fecundate his own wife after he has been reduced to a corpse and produce legitimate children after his death" [1]. However, sperm cryopreservation did not become a realistic proposition until the discovery in 1949 of the potent cryoprotective properties of glycerol [2]. The first human births resulting from artificial insemination of cryopreserved semen were reported by Bunge and Sherman in 1953 [3]. Since that time, many children have been born as a result of this rather simple procedure of assisted reproduction. Theoretical considerations suggested that long-term cryostorage would require the use of temperatures lower than −130°C, the glassy-transition temperature, below which ice-crystal growth is inhibited [4]. Consequently, liquid nitrogen (−196°C) storage became the standard very early in the history of sperm banking [5].

Nowadays, human semen cryopreservation is an extensively performed routine technique in fertility clinics and hospitals worldwide. Sperm cryopreservation may provide the opportunity for future fertility in a variety of situations. Although semen cryopreservation has proven to be very valuable, the quality of frozen sperm is highly affected during the process.

General Aspects of Sperm Cryopreservation

It is generally accepted that the motility and fertilizing ability of frozen sperm do not correlate each other, and sublethal damages occurring within the sperm cell are thought to be

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responsible for this difference [6]. Generally, living cells undergoing cryopreservation are subjected to two major factors that are responsible for cryoinjury in a sequential manner (low temperature and crystallization of intracellular and extracellular water). These factors have deleterious effects on the sperm plasma membrane as changes in lipid composition and location [7–9]. These insults to the sperm membrane are in turn responsible for cell leakage of many intracellular components resulting in reduced sperm metabolic activities. Therefore, cytoplasmic and membrane-bound proteins and enzymes, as well as other components, are eliminated from the sperm cell [10–13]. These cryoinjuries include loss of membrane fluidity and integrity [14–16], oxidative stress leading to lipid peroxidation [17, 18], DNA fragmentation [19–21], and cytoskeleton modifications [22]. Also, freezing process causes disruption of cold-sensitive microtubule containing structures such as the meiotic spindle [23–27]. However, Donnely et al. demonstrated that sperm frozen unprepared from seminal fluid appears to be more resistant to freezing damage than frozen prepared sperm either by Percoll density centrifugation or a direct swim-up procedure and frozen in seminal plasma. In fact, although progressive motility is significantly greater in fresh prepared sperm compared with fresh unprocessed semen, prepared sperm suffer a greater decrease in progressive motility than raw semen after freezing. In addition, progressive motility is significantly improved by preparation of thawed semen, although the sperm velocity does not change. Progressive motility may be significantly improved by freezing prepared sperm in seminal plasma, although values are still significantly lower than that of the fresh samples. This is again due to the presence of seminal plasma because human sperm are particularly sensitive to free-radical assault due to their high content of polyunsaturated fatty acids and lack of repair mechanisms [28]. Further improvements can be achieved by selecting out the subpopulation of sperm with best motility and DNA integrity and freezing these sperm in seminal plasma, making this the optimal procedure. Therefore, freezing sperm in seminal plasma improves motility and DNA integrity after thawn [28].

Anyway, cryopreservation of human sperm remains an essential tool for the preservation of male fertility. Freezing of sperm before initiation of treatment provides to patients a type of "fertility insurance" and may allow them to father their own children through the use of in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI). On the other hand, human spermatozoa have unusual cryobiological behavior, and improvements in their survival have not been achieved by the standard approaches of cryobiology. In fact, despite continuous methodological optimization, the process of cryopreservation and thawing leads to an activation of apoptosis signal transduction in a certain amount of the cryopreserved spermatozoa probably contributing to the reduction of the fertilizing capacity [29–31]. In fact, the mitochondria are preferentially susceptible to apoptotic stimuli due to their compartmentation within the midpiece region [32, 33].

As far as sperm membrane damage is concern, the susceptibility of the sperm of other mammal species to cryodamage during the freezing process appears to be related to a high ratio of saturated vs. unsaturated fatty acids, together with low cholesterol content [34]. Interestingly, human sperm membranes have unusually high cholesterol contents, and these high levels are known to stabilize membranes during cooling [35]. Recently, a study showed that higher cholesterol contents do not appear to protect sperm against cryodamage. Conversely, calcium equilibrium appears to be essential for a good postthaw recovery. On the other hand, mitochondrial activity is not reflecting the possibilities of sperm survival and is probably not a good indicator of the sperm metabolism. Although further experiments are needed to improve postthaw recovery, some calcium chelators (i.e., EDTA) or determinate phospholipids, which would prevent calcium flux into the sperm, may be added to the sperm freezing medium. Although ICSI may be used for men with severe sperm pathologies and the generation of poor results after the thawing of sperm, there are some specific cases where an improvement in the freezing methods are essential, such as in management of sperm donor samples [36].

Sperm membrane integrity becomes impaired with translocation of phosphatidylserine from the inner to the outer leaflet of the sperm plasma membrane [37, 38]. This translocation is considered one of the early signs of terminal phase of apoptosis [39]. The specific binding of annexin V to phosphatidylserine can be used for detection [40] and magnetic separation of spermatozoa with disturbed plasma membrane [41].

According to Paasch, sperm freezing showed a significant overall activation of caspases with a decrease in mitochondrial membrane potential. Both mature and immature fractions had significant activation of caspases followed by a decreased number of sperm with intact mitochondrial membrane potential after cryopreservation. In addition, cryopreservation and thawing induce caspase activation in mature annexin V1 in ejaculated human spermatozoa as well as in immature annexin V1 and annexin V2 sperm. Cells without susceptibility to apoptosis activation can thus be separated by magnetic field by magnetic cell sorting (MACS) technique, which may prove to be of clinical relevance [42]. In fact, the separation of a distinctive population of nonapoptotic spermatozoa with intact membranes may optimize the cryopreservation–thawing outcome. Magnetic-activated cell sorting using annexin V microbeads enhances sperm motility and cryosurvival rates following cryopreservation [43]. However, MACS using annexin V microbeads enhances the percentage of spermatozoa with intact transmembrane mitochondrial potential and mitochondrial integrity survival rates following cryopreservation [33].

The change in the plasma membrane selective permeability caused by cold shock induces the loss of many components from the sperm cell resulting in reduced metabolic activities. During this process, cytoplasmic and membrane-bound proteins are lost [44–46]. Recently, a decrease in P25b and P34H (fertility markers according to Syllivan) after human sperm cryopreservation has been shown. However, this decrease in P34H detection is most likely a consequence of cryopreservation-induced damage to the sperm plasma membrane rather than total cell damage. Due to the fact that sperm deficient in P34H are unable to bind to the egg's zona pellucida (ZP) [47], lower levels of P34H observed in cryopreserved semen may correlate with the loss of fertility after freezing–thawing of human sperm.

According to Morris et al., viability on thawing does not appear to correlate with conventional theories of cellular freezing injury, suggesting that, for human spermatozoa, other factors determine viability following freezing and thawing. Improved methods of cryopreservation may be developed by specifically manipulating the manner in which cells experience physical changes instead of imposing a linear temperature reduction. Treatments which followed a chosen nonlinear concentration profile, referred to as "controlled concentration," allowed recovery of almost all the cells which were motile before freezing [48].

Effect of Storage Temperature on Sperm Cryopreservation

The standard method for preservation of human sperm is storage in liquid nitrogen at a temperature of −196°C [2]. A number of studies have documented that improvements in cooling technique and the use of improved cryopreservatives, as well as thawing at 37°C in a water bath, improve postthaw sperm quality [3]. It appears, however, that the loss of sperm motility with the cryopreservation process does not increase with prolonged periods of cryopreservation, making long-term storage feasible. In fact, it has been reported that the semen of six donors was stored for 28 years suggesting that it may be possible to store human sperm virtually indefinitely if it is kept under liquid nitrogen. This is a pertinent information for clinicians to refer pubescent boys and young men for sperm banking before chemotherapy. Some of these young men may require sperm storage for long periods (years) [49–52].

Sperm cryopreservation in liquid nitrogen at −196°C has become standard protocol in most andrology facilities. Because this technique is pretty much acceptable everywhere, little consideration has been given to the potential effectiveness of short- or long-term storage of sperm at higher temperatures. Unfortunately, not every place has consistent access to liquid nitrogen cryopreservation facilities. Trummer et al. showed that sperm storage at −70°C is linked with a greater loss of sperm motility than is storage at −196°C. The reduction in motility noted at 1 week, however, was fairly modest. Unfortunately, the loss of sperm motility increased dramatically after 3 months of storage. These results indicate that short-term storage of sperm at −70°C may be a viable option if liquid nitrogen storage facilities are not available. Long-term storage at this temperature (70°C), however, appears to be linked with an unacceptable reduction in sperm motility [53].

Even though the standard protocol of sperm storage at −196°C is widely used in almost every center, the technical aspects of freezing and thawing sperm and preparing cryopreservation media have been refined over the years [54–57]. Various methods of cryopreservation have been evaluated for their effects on sperm motility [58–60]. However, the methods for freezing and thawing semen that optimize motility recovery have not been firmly established. In addition, the optimum rate of temperature drop during freezing remains controversial [54, 58, 59]. The flash-freezing technique in which the sample is plunged directly into liquid nitrogen produces sperm recovery rates that are comparable to those seen with computer-controlled, slow-staged freezing [60]. In addition, a variety of cryoprotectants are available to protect sperm from the negative effects of the cryopreservation process. Many andrology laboratories use TES and Tris yolk buffer (TYB; Irvine Scientific, Santa Ana, CA), but some of the other media commonly used are the following: Sperm Freezing Medium (Medi-Cult, Copenhagen, Denmark) and Enhance Sperm Freeze (Conception Technologies, San Diego, CA). However, a recent study by Nallella et al. showed that Irvine Scientific method is associated with more flash freezing compared with the Cleveland Clinic Foundation method and results in better preservation of sperm motility and a higher cryosurvival rate. According to their study, TES and TYB is most effective at protecting sperm from the negative effects of the cryopreservation process [61]. This may be due to the presence of egg yolk along with glycerol.

The advantages of the fast-freezing and slow-staged cooling methods have long been debated. Studies have reported results in favor of both the fast-freezing method [62] and the slow-staged cooling method [63–65]. A recent study showed that there was no difference in sperm quality preservation when semen samples is frozen by fast-freezing technique or by slow, controlled freezing method, either in liquid nitrogen or vapor-phase nitrogen [66].

Another study has showed that freezing spermatozoa in alginic acid microcapsules resulted in an increase of immobilized spermatozoa by 18.3%, compared with the standard protocol. The method of cryopreservation of small amounts of spermatozoa is a feasible and easy method that does not demand special laboratory equipment. Because microcapsules are dissolved under microscopic control, a 100%

recovery rate of spermatozoa is estimated. Furthermore, microencapsulation of spermatozoa excludes the possibility of contamination with foreign material, either spermatozoa or genetic material. With microcapsules, the recovered spermatozoa can be divided into samples and cryopreserved separately for consecutive ICSI procedures. This gives the opportunity to collect spermatozoa, independently from oocyte recovery and ICSI, and assures the availability of spermatozoa on the day of ICSI [67].

Cryopreservation of Human Spermatozoa Within Oocyte Empty Zona

Men who have azoospermia can now be treated through the surgical isolation of spermatozoa using microsurgical epididymal sperm aspiration (MESA) or testicular sperm extraction (TESE) [68]. However, multiple testicular operations are not only costly but may lead to adverse physiologic effects and possible testicular failure [69]. The need to repeat these procedures can be avoided by the use of sperm cryopreservation [70]. Cohen et al. were the first to demonstrate that the ZP is an ideal vehicle for sperm cryopreservation [71]. After removal of the cellular material from oocytes or embryos, the empty ZP provides a suitable vehicle for preserving the few spermatozoa that can be obtained from patients with severe male infertility [71]. Cryopreservation in ZP avoids the loss of sperm that occurs with the dilution and washing of sperm during conventional cryopreservation procedures [72]. The rate of recovery of motile sperm after cryopreservation in ZP is higher than after conventional cryopreservation with cryoseeds and dithiothreitol [72–74]. With cryopreservation in ZP, TESE or MESA could be performed without regard to the timing of egg retrieval [75].

Sperm Cryopreservation in Specific Situations

Obstructive Azoospermia

Obstructive azoospermia is caused by several different etiologic processes. Ejaculatory duct obstruction, vasectomy, postinfection obstruction, and congenital bilateral absence of the vas deferens are some of the major causes of obstruction. An important approach in management of patients with obstructive azoospermia is retrieval of sperm for various assisted reproductive technology (ART) procedures through MESA, TESE, and percutaneous epididymal sperm aspiration (PESA).

In general, spermatozoa from epididymis are considered more mature than the testicular spermatozoa and provide a higher pregnancy rate [76]. Often, it is not possible to retrieve spermatozoa from the epididymis. In some cases, there might be a complete absence of epididymis. TESE is the modality of choice in the management of nonreconstructable obstruction of the excurrent duct system, when epididymal sperm aspiration is not available or unsuccessful. It is possible to extract a large number of spermatozoa from the epididymis or testicular tissues of patients with azoospermia of obstructive etiology. In fact, pregnancy outcome in obstructive azoospermia using these spermatozoa is higher than in nonobstructive azoospermia [77]. Only a small portion of these spermatozoa are needed for IVF/ICSI techniques, and the remaining tissue can be divided into several aliquots and cryopreserved for subsequent use. After carrying out a TESE procedure, the testicular tissue can be divided into several aliquots and cryopreserved. This would favor multiple ICSI cycles [78], thereby avoiding further surgical biopsies to retrieve spermatozoa in the future [79]. Generally, the time from biopsy to processing and freezing is within 1 and 1.5 h; however, a recent case report showed pregnancy even with the interval between the biopsy and the testicular tissue cryopreservation up to 15 h. Therefore, for many programs, the use of cryopreserved testicular tissue avoids the need for fresh testicular tissue at the time of ICSI [51].

Nonobstructive Azoospermia

In contrast to obstructive azoospermia, where viable sperm can easily be retrieved from the frozen specimens, the impaired quality of the testicular tissue present in nonobstructive azoospermic (NOA) patients does not allow for cryopreservation and later use for ICSI in all cases. As has been demonstrated for ejaculated sperm, a significant decrease in sperm motility and viability by freezing and thawing also occurred for testicular sperm [80]. This implies that cases with extremely low numbers of sperm retrieved can hardly be considered candidates for cryopreservation.

Even in a program with low-restrictive criteria for patient allocation and cryopreservation of testicular sperm, diagnostic testicular sperm retrieval followed by cryopreservation may be the procedure of choice. In order to counteract the reasonable risk of not finding sperm or only immotile sperm, scheduling fresh surgery as backup or counseling the couple for donor sperm as backup is recommended. The use of totally immotile sperm after thawing should be discouraged on the basis of the present data [81].

Patients with Cancer

Recent advances in the diagnosis and treatment of malignant diseases has brought into focus certain quality-of-life issues, such as the problem of infertility [82]. The impact of these problems is magnified in malignant diseases that predominantly

affect patients in the reproductive age group. An increasing number of people are being successfully treated for cancer, and for those with an expectation of long-term survival, the late effects of treatment are of concern. In fact, in the past, cancer survivors tended to be most concerned about disease recurrence and treatment side effects. As survival rates have increased, however, patients are now also concerned about quality-of-life issues such as preserving fertility potential [83].

Depending on the underlying disease, the age of the oncological patient, the type of therapeutic agent used to treat the cancer, the cumulative doses used, and the duration of the treatment, 10–100% of surviving cancer patients will show reduced semen parameters after their cure. An average of 15–30% of cured cancer patients remain sterile in the long term [84].

The degree of testicular damage is drug specific and dose related. Ninety-seven percent of men with Hodgkin's disease (HD) who were treated with MOPP (mechlorethamine, vincristine, procarbazine, and prednisone) became azoospermic compared with 54% of those treated with ABVD (adriamycin, bleomycin, vinblastine, and dacarbazine). Only 14% of those in the MOPP group recovered spermatogenesis compared with 100% in the ABVD group [85]. Men with testicular germ cell cancer can expect irreversible impairment of the gonadal function at cumulative cisplatin doses 400 mg/m^2 [86]. In the case of irradiation to the adult male testis, permanent azoospermia can be induced by doses in excess of 400 cGy, whereas recovery of spermatogenesis is seen at doses of 300 cGy or below [87].

According to a study by Spermon et al., before the cancer being diagnosed, 79 (66%) of 120 couples who attempted to conceive succeeded within 1 year. After treatment, 38 (43%) of 88 couples conceived within 1 year. Seven couples used cryopreserved sperm to conceive a child after treatment. The different treatment modalities do not significantly influence the outcome of patients' wish for children. Congenital malformations were recorded in approximately 4% of the children born before or after treatment. Therefore, although the majority of the patients with testicular cancer have a fulfilled wish with regard to children, it seems to be more difficult to father a child after treatment compared with the case in the general population. Because it is not possible to predict which patient will have fertility problems after treatment, cryopreservation should be offered to every testicular cancer patient. In addition, an increased risk for congenital malformations is not observed [88].

It is recommended that all male cancer patients up to age 55 (at least or according to institutional criteria) be referred for sperm cryopreservation. The age of 55 years is the maximum recommended age for freezing sperm according to the Human Fertilisation & Embryology Authority (HFEA) regulations [89]. Further deterioration have been observed following cancer treatment, and this is of a major importance to young patients. Sperm quality following cancer treatment depends on many factors: initial sperm characteristics, type of cancer, and therapeutic approach. Unfortunately, it is impossible to predict who will have normal spermatogenesis and who will become azoospermic.

In many cancer subjects, sperm quality is already reduced before receiving any treatment. Studies have been shown that semen quality in adolescent male patients with cancer found that semen parameters may be diminished, and sperm cryopreservation during potentially sterilizing treatment should be considered [90–92]. At present, sperm banking remains the only proven method, although hormonal manipulation to enhance recovery of spermatogenesis and cryopreservation of testicular germ cells are possibilities for the future [5, 93–95]. According to Hallak et al., patients with testicular cancer, leukemia, and lymphoma had the worst seminal quality compared to other cancer groups [96, 97]. Patients with malignant diseases in general have lower total motile sperm count and motility compared to normal semen donors. Furthermore, and of utmost importance, the authors concluded that prefreeze or postthaw semen quality in cancer patients is not affected by the type of disease. According to Gandini et al., the recovery of spermatogenesis after chemotherapy or radiotherapy in their group of testicular cancer patients studied was not a function of pretherapy sperm parameter quality [93]. Therefore, cryopreservation of semen should be offered to cancer patients irrespective of the type of the disease [5, 98–100].

The intersection of cancer and reproduction raises ethical issues for both cancer and fertility specialists, including issues of experimental vs. established therapies, the ability of minors to give consent, the welfare of expected children, and posthumous reproduction. In some respects, cancer-related infertility is not markedly different than other kinds of infertility. In other way, however, the context of cancer gives rise to issues of patient and offspring welfare that do not arise in other infertility settings. Reproductive physicians play important roles in helping to preserve the reproductive capacities of young cancer patients. First, they are involved in developing and using procedures to preserve gametes, embryos, and gonadal tissue before treatment. Second, fertility specialists will assist cancer survivors in using preserved gametes and tissue or in providing other assistance in reproduction [101].

Men who once had little or no chance of establishing a pregnancy through assisted techniques now have fertility rates approaching that of a couple undergoing standard IVF when there is no male infertility involved. However, according to Zapzalka et al., most of the oncologists (74%) are unaware of recent advances in reproductive technology in which only a few sperm are needed for successful IVF with ICSI [102]. This lack of awareness may be contributing to underutilization of sperm cryopreservation by male cancer

patients. Coincidentally, the number of patients that the oncologists estimated to actually cryopreserve sperm is also very small (27%). This leads to speculation that if more oncologists knew of the existence of ICSI, the percentage of patients who cryopreserve sperm might increase.

According to Schover et al., 19% of the 283 survivors of cancer from the Cleveland Clinic Foundation tumor registry had significant anxiety that their cancer treatment could affect negatively their children's future, and only 57% received information from their healthcare providers about infertility after cancer [103].

Other reproductive concerns are discussed even less often. For instance, patients with cancer may have chromosomal abnormalities in the malignant cell of origin and an increased frequency of human sperm chromosomal abnormalities after radiotherapy has been reported. However, reassuringly, studies have shown that children born after completion of cancer treatment have no increased risk for chromosomal abnormalities or birth defects before or after treatment.

Intraoperative Sperm Harvesting During a Vasectomy Reversal and Cryopreservation

The availability of ICSI technique has encouraged some surgeons to offer cryopreservation of sperm that may be harvested during vasectomy reversals. Cryopreservation of sperm during vasoepididymostomy is especially important because of a reported 35% rate of azoospermia after microsurgical vasoepididymostomy [104]. Other investigators have reported that motile sperm were present in the intraoperative vasal and epididymal fluid in 35% of 603 vasectomy reversals [105]. However, performance of a vasal or epididymal anastomosis should be prioritized over sperm harvesting during vasectomy reversals. The surgeon should perform the reversal at the location farthest from the testicle where intact sperm, regardless of their motility, are present, rather than closer to the testicle in order to harvest motile sperm. Before sperm harvesting and cryopreservation are performed, the patient and his partner should assess the cost effectiveness of, and their ability to afford, IVF/ICSI. When harvesting sperm during vasectomy reversals, surgeons must alert laboratory personnel to cryopreserve small aliquots of sperm that are appropriate for later use with ICSI rather than larger aliquots for either vaginal or intrauterine insemination (IUI) or IVF without ICSI [106, 107].

Prior to the Absence of the Partner

If it is anticipated that the male partner will be unavailable during the optimal time for the procedure, it is possible to cryopreserve a semen sample prior to his departure as a backup in the event he is prevented from providing a sample at the time of the procedure. While this may not be optimal, it can often keep the time and finances invested in a procedure from being wasted due to unforeseen circumstances [108].

Assisted Reproduction

Since the introduction of a method for freezing human semen, the indications for sperm cryobanking have been greatly expanded by recent breakthroughs in assisted reproduction, for one's own future use and donor banking [5]. However, it has been shown that the process of freezing and thawing is related with a variable loss of sperm quality [109].

Although the adverse effect of freezing and thawing upon sperm quality is even more pronounced in cases of poor semen quality, with the advent of ICSI, only a small number of motile spermatozoa are required for a successful fertilization [110, 111]. In fact, ICSI can be performed with fresh and cryopreserved spermatozoa from ejaculated semen from patients with oligoasthenoteratozoospermia (OAT) or from spermatozoa extracted from the epididymis or testis in cases of obstructive or nonobstructive azoospermia [112].

It is well known that IUI with cryopreserved sperm results in a lower pregnancy rate compared to fresh sperm, but many studies have been showing that fertilization and pregnancy rates of ICSI using cryopreserved spermatozoa are similar of freshly obtained sperm [113–117].

Even though there is no doubt that cryopreserved spermatozoa can be used to fertilize oocytes by ICSI, more studies comparing the efficacy of ICSI with either fresh or cryopreserved ejaculated spermatozoa from infertile patients should be performed. Also, the majority of studies comparing fresh and cryopreserved sperm have shown results from sperm surgically retrieved [114–117]. Borges et al. demonstrated that when the semen sample had motility decreased, the fertilization rate is higher with fresh sperm than with cryopreserved sperm. However, the implantation and pregnancy rates miscarriage rates are similar. This finding corroborates the idea that the cryopreservation process may cause more damage to patients with asthenozoospermia than patients with normal semen analysis or oligozoospermia. In fact, it is postulated by the authors that the worst normal fertilization rate detected in men with asthenozoospermia compared to normozoospermic or oligozoospermic men could be because the semen with low motility may have abnormalities in the sperm even before the cryopreservation, and the damage caused by the cryopreservation could be much higher than detected in other sperm characteristics [112].

According to a study published by Schmidt et al., following antineoplastic treatment, 43% of the men had motile

spermatozoa in the ejaculate, but 57% were azoospermic. A total of 151 ART cycles were performed [55 IUI, 82 ICSI, and 14 ICSI–frozen embryo replacement (FER)]. Their clinical pregnancy rate per cycle was 14.8% after IUI, 38.6% after ICSI, and 25% after ICSI–FER. The corresponding delivery rates were 11.1, 30.5, and 21%. Cryopreserved semen was used in 58% of the pregnancies. The delivery rate per cycle was similar after using fresh or cryopreserved spermatozoa. Therefore, male cancer survivors have a good chance of fathering a child by using either fresh ejaculated sperm or cryopreserved sperm [118]. Recently, Agarwal et al. [94] reported the outcome of ART in 29 male cancer survivors all using cryopreserved semen. A total of 87 cycles were performed with a mean pregnancy rate of 18.3% per cycle (7% after IUI, 23% after IVF, and 37% after ICSI).

Male cancer patients should be encouraged to freeze numerous sperm samples even when sperm count and motility are poor [119]. In these cases, ICSI is a powerful technique compared with intrauterine injection since thawed sperm samples with poor parameters can produce relatively high fertilization rates resulting in normal pregnancies and deliveries. The possibility to repeat treatments even in face of a limited number of sperm samples appears to be of utmost importance.

There are concerns that men with cancer who initiate a pregnancy either before or after treatment may have children who are at an increased risk for congenital anomalies. However, Hansen et al. studied the rate of congenital abnormalities in children born to fathers with cancer before treatment and observed a congenital malformation rate of 3.8%, which is comparable to the general population [120]. Redman et al. found no congenital abnormalities in three children who were born using cryopreserved semen from patients with Hodgkin's disease [121]. Moreover, Spermon et al. reported a 4% rate of congenital malformations in children born before or after treatment of testicular cancer, compared with 2.2% in the general population [89]. Thus, cancer patients should be informed that, currently, there is no available evidence for increased incidence of congenital abnormalities in children.

In recent years, some physicians have raised doubts regarding the justification and necessity of providing the facilities for banking spermatozoa before cancer treatment because of the relatively small number of men who used it following completion of treatment and consequently the small number of children born as a result of cryopreserved spermatozoa. Indeed, it seems that 5–15% of the patients who banked their semen before treatment return for fertility purposes. The reasons for that are: short period of original disease, anxiety about potential risks for the children, and uncertainty about their long-term health and recovery or waiting for possible recovery of gonadal function. Hallak et al. surveyed 56 patients at the Cleveland Clinic who requested to discontinue storing of spermatozoa, and they concluded that most patients decided to discontinue sperm banking because they either regained fertility or had improved semen quality [122]. Even if properly counseled, not all patients will eventually bank semen before their treatment. One study reported that only 42% of appropriately counseled patients did bank their semen to counter sterility [123], while another recent study reported a value of 54% [124].

The Cleveland Clinic survey revealed that, of those currently childless, 76% wanted children in the future [125]. Moreover, approximately 80% of the patients viewed themselves positively as actual or potential parents. Addressing this issues immediately upon diagnosis helps young cancer patients and their families to face the disease and cope with treatment in a more optimistic light. Currently, all male cancer patients of reproductive age who will have treatment that may affect testicular function and who may desire children in the future should cryopreserve sperm before the initiation of therapy. It is vital therefore to keep records of patients having postcancer infertility treatment and to monitor the children born as a consequence of these treatments.

While many oncologists now tend to use less gonadotoxic treatments, semen cryopreservation should always be offered to each cancer patient since recovery of spermatogenesis cannot be guaranteed for the individual patient, because of important interindividual variances or because a therapeutic regimen may be started with limited gonadotoxicity, but eventually a more gonadotoxic therapy may be indicated because of treatment failure.

Sperm Cryopreservation in Boys

Another point of interest is the prepubertal boys with malignant disease. Spermatozoa can be obtained by masturbation from about the age of the 14 years. To preserve fertility, it is necessary to determine the risk of fertility impairment before instituting cancer therapy. Predicting the impact of treatment on reproductive function in individual children based on expected exposures is notoriously unreliable. Current tools, both biochemical and biophysical, are unsuitable for assessing actual reproductive impacts in prepubertal and peripubertal children. Even when pubertal onset and progression is apparently normal, the integrity of gametes may have been compromised.

Cryopreservation of semen and subsequent IVF is the only standard option for postpubertal males, and spermarche is the watershed around which options for boys are defined. Spermarche typically is an early to midpubertal event and occurs before the ability to achieve ejaculation [126]. In the mature adolescent, semen is usually obtained by masturbation, with electrostimulation or vibratory stimulation as

alternatives (the latter two may be applicable in peripubertal boys). However, the rate at which viable samples are obtained is highly variable. These adolescents are often sick as well as embarrassed and uncomfortable. One study of 62 attempts by adolescents to bank sperm before therapy resulted in totally normal semen in only four [127]. Adolescents may be more successful if unaccompanied by parents [92, 128].

Testicular tissue cryopreservation would be an important technique for fertility preservation in prepubertal boys, who do not yet have sperm in the ejaculate and who are scheduled to undergo gonadotoxic treatment [129]. If the boy already has spermatozoa in his semen, it is best to freeze sperm [92, 130]. Cryopreservation of testicular cell suspension has been proposed as an alternative method suitable for patients with azoospermia and young patients who are not yet producing sperm [131]. Banking of testicular tissue in prepubertal boys before gonadotoxic treatment is a crucial step in fertility preservation [129]. Testicular biopsies for fertility preservation are therefore ethically justified [132]. However, malignancy recurrence prevention is an important prerequisite for any clinical application of testicular stem cell transplantation. Therefore, the storage of prepubertal testicular tissue is currently emerging as a potential solution [92, 129, 133–135]. After being cured, the frozen–thawed tissue may theoretically be transplanted [133, 135], xenotransplanted [136, 137], or matured in vitro [138, 139]. So far, the testicular stem cell transplantation has provided the most promising results in animal models, mostly murine models. Also, ethically, autologous testicular stem cell transplantation may be more acceptable than xenotransplantation strategies.

Anyway, for pubertal boys, who are too young to ejaculate, cryopreservation of testicular biopsy and TESE after cryopreservation might be an option for fertility preservation [140]. For prepubertal boys, when full spermatogenesis is not yet ongoing, it is essential to store the spermatogonia and the neighboring cells as undamaged integrated tissue [141]. Preservation of Sertoli cells and cell-to-cell contacts in testicular tissue have proved to be important for subsequent maturation of spermatogonia [142].

A study by Kliesch et al. demonstrated that adolescent patients, aged 14–17 years, are good candidates for semen banking [130]. In a large series, Bahadur et al., reported that 86% of 238 boys of postpubertal age up to 19 years old could produce a semen sample for cryostorage [92]. In four out of the remaining 33 boys, eventually, sperm recovered from a urine sample was cryopreserved [92]. A study involving 45 male adolescents showed that for 20 of them (44.5%), semen cryopreservation was not performed because they were not judged as being mature enough to deliver a semen sample by masturbation. Another four boys failed to deliver a semen sample because of masturbation problems and finally, for two boys, semen was collected by alternative methods such as penile vibrostimulation or electroejaculation, both performed under general anesthesia [143]. Electroejaculation in adolescents may be an alternative to masturbation in order to obtain semen for cryostorage [144, 145].

Concluding, the safety and effectiveness of the ART are essential issues. It is important to have ethics approval and strong motivation for all procedures included in programs for fertility preservation in young patients [100, 132, 146, 147]. Children and their parents should be informed about the experimental stage of the study. However, it is important to inform all patients facing infertility as a side effect of gonadotoxic chemo- and radiotherapies about the options available to preserve their future fertility [148–150].

Another interesting situation about cryopreservation is the one involving boys with cryptorchidism. Boys with cryptorchidism often face fertility problems in adult life despite having orchiopexy performed at a very young age. During this operation, a biopsy of the testis is normally taken in order to evaluate their infertility potential and the presence of malignant cells. Evaluating biopsies from 11 testes (eight boys), one fresh and two cryopreserved pieces were cultured for 2 weeks and prepared for histology. The morphology of the fresh and frozen–thawed samples were similar, with well-preserved seminiferous tubules and interstitial cells. A similar picture appeared after 2 weeks of culture, but a few of the cultured biopsies contained small necrotic areas. The presence of spermatogonia was verified by c-kit-positive immunostaining. Production of testosterone and inhibin B (ng/mm^3 testis tissue) in the frozen–thawed pieces is on average similar to that of the fresh samples [151]. Therefore, Kvist et al. concluded that intact testicular tissue from young boys with nondescended testes tolerates cryopreservation with surviving spermatogonia and without significant loss of the ability to produce testis-specific hormones in vitro. It may be an option to freeze part of the testis biopsy, which is routinely removed during the operation for cryptorchidism, for fertility preservation in adult life.

Sperm DNA Damage and Cryopreservation

An intact chromatin structure is extremely important for the fertilizing ability of the sperm, and it has been shown that defective spermiogenesis is associated with abnormal remodeling of sperm chromatin and membrane components, which in turn results in morphologically abnormal spermatozoa. Sperm chromatin structure, once believed to be stable during the cryopreservation process, is now found to be altered during freezing–thawing of spermatozoa. In addition, cryopreservation appears to reduce the ability of sperm chromatin to decondense during fertilization, suggesting the detrimental effect of freezing–thawing on sperm chromatin.

Recently, several groups have reported the effect of cryopreservation on sperm DNA integrity [152–158]. Although

in some reports there appears to be an increase in sperm DNA fragmentation after thawing, in other studies, no such effect was found [152–158]. It has been demonstrated by Toro et al. that processing of semen for ART, including incubation and cryopreservation of semen, may result in increased sperm DNA fragmentation. Therefore, unnecessary incubation of semen in the laboratory should be avoided. In fact, cryopreservation has deleterious effects on sperm DNA by inducing DNA fragmentation and oxidation, but the mechanisms underlying such damages need to be elucidated by further investigations. Cryopreservation induces apoptotic sperm DNA fragmentation in men, regardless of sperm concentration. Men with oligospermia present with higher precryopreservation and postcryopreservation apoptotic sperm DNA fragmentation [155]. In fact, they noticed the same increase in DNA fragmentation in normospermic as well in oligospermic infertile patients [155]. Interestingly, in a very elegant study, Adiga et al. found a strong correlation between the amount of initial sperm DNA damage and postthaw survival [156]. However, no significant enhancement in DNA damage was observed by the cryopreservation of spermatozoa with several amounts of DNA damage. Neither the presence nor the type of cryoprotectant affects the DNA integrity of spermatozoa after cryopreservation and separation using density gradient centrifugation [158]. Individuals with lower prefreeze fragmentation in separation using density gradient centrifugation have larger increases in fragmentation and are less likely to exhibit lower levels of fragmentation postthaw [158].

Step by Step Protocol of Laboratory Procedures

Equipment and Reagents

- Microcell counting chamber from Conception Technologies
- 5-mL Eppendorf pipette
- Aliquot mixer
- Vortex
- –20°C Freezer
- LN2 container with racks
- Sterile specimen container
- Sterile 15-mL centrifuge tubes with caps
- Sterile serological pipettes (1, 2, and 5 mL capacity)
- Sterile Nunc cryovials (1 and 2 mL capacity)
- Colored cryomarkers
- Test tube racks (for 15-mL test tubes)
- Cryovial racks
- Stainless steel canes for cryovials
- Plastic cryosleeves
- Cryogloves
- Latex gloves
- 37°C Incubator
- LN2 from supplier
- Eosin–nigrosin stain
- Microslide
- Coverslips
- Sperm washing media
- Makler chamber
- Freezing medium (Test yolk buffer with glycerol; TYB-G) from Irvine Scientific, Sabta Ana, CA
- Sperm washing media (HTF): Enhance-W from Conception Technologies, La Jolla, CA

Procedure (Aspiration from Surgery)

Technical note: Sterile techniques should be used throughout specimen processing. Gloves are mandatory for all procedures dealing with body fluids. Latex, however, may be toxic to sperm. Therefore, care should be taken to prevent contamination of the specimen with latex or talc. Vinyl gloves are an available alternative.

1. Cryopreservation worksheet labeled with the patient name, diagnosis, and LN2 container and rack. If there is more than one specimen, then have a worksheet for each specimen.
2. Every time a laboratory receives the notice that an aspirate is ready, take one bottle of frozen test yolk media and put into the 37°C incubator to thaw.
3. A technologist will go to the surgery room to retrieve the specimen(s). Keep the vials warm in your hands.
4. Normally, two to three specimen will arrive from surgery. Make sure the name of the patient as well as the specimen number.
5. Using sterile technique, measure the volume of each specimen and record on the appropriate cryopreservation worksheet.
6. Centrifuge the specimen in the original container for 5 min at 1,600 rpm.
7. Label three sterile 15-mL conical centrifuge tubes with the patient's name and specimen number.
8. Transfer the supernatant of specimen 1 and specimen 2, and if there is specimen 3 also in the "supernatant" tube. Both supernatants are now combined into one tube.
9. Add 0.5 mL HTF (sperm washing media) to each pellet to resuspend. Mix gently.
10. Transfer a drop of each specimen into a prelabeled conical cup for semen analysis.
11. Perform the following on each specimen aspirate: regular manual semen analysis, Endtz test (leukocytospermia test), eosin–nigrosin stain procedure, Tygerberg's strict criteria form morphology evaluation.
12. Always notify if the surgeon found no motile sperm on the wet prep.

13. Within 1 h of specimen collection, add an aliquot of freezing medium equal to 25% of the resuspended aspirate volume to the centrifuge tube with a sterile pipette.

    Note: Since the resuspended volume for each aspirate is 0.5 mL, then divide 0.5 by 4 to obtain 0.13. Therefore, add 0.13 mL of freeze media four times to the specimen.
14. Gently rock the specimen(s) with the freezing media for 5 min on an aliquot mixer.
15. Repeat steps 13 and 14 three times or until the volume of freezing media added is equal to the specimen volume in step 9.
16. Centrifuge the tube labeled "supernatant" for 5 min at 1,600 rpm.
17. Remove the supernatant from the "supernatant" tube. Resuspend the pellet with 0.5 mL of HTF (sperm washing media). Mix gently. Remove one drop for a semen analysis.

    Note: If no motile sperm is found in the "supernatant" tube, then do not start freezing. If motile sperm is found, then freeze as in steps 13–15.

    During the mixing steps above, use appropriately colored cryomarkers to label 2-mL cryovials and canes. The volume added to the vial should not exceed 1.8 mL per vial.
18. Label an additional 1.0-mL cryovial as in for each specimen. This will contain a leftover aliquot of the cryodiluted specimen to be assessed of cryosurvival 24 h after freezing in LN2.
19. A visual inspection should therefore be made of the cryodiluted specimen for motility. A manual motility can be done using a Microcell chamber or a Makler chamber and a Nikon 2-phase microscope. The percent motility should be documented on the cryopreservation worksheet under cryodilution motility.
20. Distribute the well-mixed, cryodiluted semen into prelabeled vials using a 1- or 2-mL sterile serological pipette. Add at least 0.2 mL to the smaller 1.0-mL cryovial.
21. Place labeled vials into a plastic freezing rack along with canes and cryosleeves and put into a −20°C freezer for 8 min. Do not open the freezer in any circumstance during this incubation.

    Note: Exposure to freezing conditions should occur within 1.5 h of specimen collection.
22. After the 8-min incubation, remove the rack and canes from the 20°C freezer. Place a maximum of two cryovials into bottom slots of canes upside down. Put into cryosleeves.
23. After a minimum 2-h incubation in liquid nitrogen vapors, turn cases upside down, immerging them into liquid nitrogen.
24. After a minimum of 24 h in liquid nitrogen, thaw the aliquot in the 1.0-mL cryovial.
25. Using cryogloves, remove cane containing the vial and snap it out. Loosen the cap and place in the 37°C incubator for 20 min.
26. Mix the vial well and analyze manually.
27. Record the cryosurvival area of the cryopreservation worksheet.
28. Assess cryosurvival in the formal:

$$\frac{\%\ \text{motility of postthaw specimen}}{\%\ \text{motility of prefreeze specimen}}$$

Procedure (Semen from Ejaculate)

Technical note: Sterile techniques should be used throughout specimen processing. Gloves are mandatory for all procedures dealing with body fluids. Latex, however, may be toxic to sperm. Therefore, care should be taken to prevent contamination of the specimen with latex or talc. Vinyl gloves are an available alternative.

1. Cryopreservation worksheet labeled with the patient name, diagnosis, and LN2 container and rack. If there is more than one specimen, then have a worksheet for each specimen.
2. Using sterile technique, measure the volume of each specimen and record on the appropriate cryopreservation worksheet.
3. Perform the following on the semen sample: regular manual semen analysis, Endtz test (leukocytospermia test), eosin–nigrosin stain procedure, Tygerberg's strict criteria form morphology evaluation.
4. Within 1 h of specimen collection, add an aliquot of freezing medium equal to 25% of the resuspended aspirate volume to the centrifuge tube with a sterile pipette.
5. Note: Since the resuspended volume for each aspirate is 0.5 mL, then divide 0.5 by 4 to obtain 0.13. Therefore, add 0.13 mL of freeze media four times to the specimen.
6. Gently rock the specimen(s) with the freezing media for 5 min on an aliquot mixer.
7. Repeat step 4 three times or until the volume of freezing media added is equal to the specimen volume in step 2.
8. During the mixing steps above, use appropriately colored cryomarkers to label 2-mL cryovials and canes. The volume added to the vial should not exceed 1.8 mL per vial.
9. Label an additional 1.0-mL cryovial as in for each specimen. This will contain a leftover aliquot of the cryodiluted specimen to be assessed of cryosurvival 24 h after freezing in LN2.
10. A visual inspection should therefore be made of the cryodiluted specimen for motility. A manual motility can be done using a Microcell chamber or a Makler chamber and a Nikon 2-phase microscope. The percent motility should be documented on the cryopreservation worksheet under cryodilution motility.

11. Distribute the well-mixed, cryodiluted semen into prelabeled vials using a 1- or 2-mL sterile serological pipette. Add at least 0.2 mL to the smaller 1.0-mL cryovial.
12. Place labeled vials into a plastic freezing rack along with canes and cryosleeves and put into a −20°C freezer for 8 min. Do not open the freezer in any circumstance during this incubation.
13. Note: Exposure to freezing conditions should occur within 1.5 h of specimen collection.
14. After the 8-min incubation, remove the rack and canes from the 20°C freezer. Place a maximum of two cryovials into bottom slots of canes upside down. Put into cryosleeves.
15. After a minimum 2-h incubation in liquid nitrogen vapors, turn cases upside down, immerging them into liquid nitrogen.
16. After a minimum of 24 h in liquid nitrogen, thaw the aliquot in the 1.0-mL cryovial.
17. Using cryogloves, remove cane containing the vial and snap it out. Loosen the cap and place in the 37°C incubator for 20 min.
18. Mix the vial well and analyze manually.
19. Record the cryosurvival area of the cryopreservation worksheet.
20. Assess cryosurvival in the formal:

$$\frac{\%\ \text{motility of postthaw specimen}}{\%\ \text{motility of prefreeze specimen}}$$

Perspectives

Although still purely experimental at this stage, testicular stem cell transplantation may provide an adequate solution to preserve the progenitive capacity of prepubertal boys. Also, even though still surrounded by complex ethical issues, cryobanking of testicular tissue from prepubertal boys may now be considered an acceptable strategy, analogous to cryobanking of ovarian cortex in young girls. However, in contrast to girls, in boys, stem cells are the target of storage, which represents an important difference in terms of potential future applications for preserving fertility.

Conclusions

Reproductive physicians play important roles in helping to preserve the reproductive capacities of young cancer patients. First of all, the reproductive physicians are involved in developing and using procedures to preserve sperm and gonadal tissue before treatment. Secondly, fertility specialists will assist cancer survivors in using preserved gametes and tissue or in providing other assistance in reproduction. The fact that the patient has just been diagnosed with cancer or survived the acute or extended phase of coping with cancer distinguishes the cancer patient from other fertility patients. Variations in type of cancer, time available to onset of treatment, age, partner status, type and dosage of chemotherapy, and risk of sterility with a given treatment regimen require that each case has its own treatment strategy. Consultation with the patient's oncologist often is essential. A key issue at the time of treatment of the cancer is whether it is medically feasible to obtain gametes or gonadal tissue for storage and later use. Questions about the patient's health and prognosis will also arise when the patient is deciding later whether to reproduce.

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Slow Freezing of Oocytes

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Giovanni Coticchio and Lucia De Santis

Abstract

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 it entails major downsides. More recently, new avenues have been opened to preserve female fertility through ART. Ovarian tissue cryopreservation and orthotopic re-implantation have shown the potential to restore ovarian function for a period of time and offer a chance for natural conception. However, so far, only very few full-term pregnancies have been achieved. Therefore, the efficacy of this approach remains to be established. Oocyte cryopreservation is powerfully emerging as an efficient ART procedure able to preserve female fertility without the implications which afflict embryo cryopreservation. Historically, control rate slow cooling has been the technological standard of human embryo cryopreservation. This has marked the early history of oocyte cryopreservation. Consistent advances of control rate slow-cooling protocols have given oocyte cryopreservation the dignity of a routine procedure in many human IVF laboratories. Progress in the last few years has been also driven by the development of the vitrification approach.

Keywords

Oocytes • Cryopreservation of oocytes • Slow freezing • Pregnancy • Control rate slow cooling in oocytes

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 it entails major downsides, such as ethical objections to the generation of conceptuses that might never be used, the need of single women to resort to donor sperm, and legal complications in case of separation of the couple. More recently, new avenues have been opened to preserve female fertility through ART. Ovarian tissue cryopreservation and orthotopic re-implantation have shown the potential to restore ovarian function for a period of time and offer a chance for natural conception. However, so far, only very few full-term pregnancies have been achieved. Therefore, the efficacy of this approach remains to be established. Oocyte cryopreservation is powerfully emerging as an efficient ART procedure able to preserve female fertility without the implications which

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afflict embryo cryopreservation. Historically, control rate slow cooling (CRSC) has been the technological standard of human embryo cryopreservation. This has marked the early history of oocyte cryopreservation. Consistent advances of CRSC protocols have given oocyte cryopreservation the dignity of a routine procedure in many human IVF laboratories. Progress in the last few years has been also driven by the development of the vitrification approach.

Methodological Aspects of Oocyte Cryopreservation

Oocyte cryopreservation, irrespective of whether CRSC or vitrification is applied, requires a rigorous system of management to control technical (type of protocol, cryopreservation media) and procedural (time of oocyte culture before and after cryopreservation, oocyte manipulation, operator experience) factors 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 necessitate of specific work areas. These areas should be adjacent to the core of the laboratory, accessible only by authorized staff, and equipped with systems to ensure sufficient air changes to prevent accumulation of liquid nitrogen vapours and monitor oxygen levels. The choice of a CRSC system should be supported by the use to two cryofreezers, to ensure safe backup and give the opportunity to run separate cycles of freezing at appropriate times, if required. Cryofreezers produce temperature graphs of the freezing 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. Dewars may be equipped with electronic monitoring systems able to send information to remote terminals, such as PC and mobile phones. Laboratory staff should be properly instructed on safety measures on the use of liquid nitrogen and have access to devices for personal protection. Quality-control and quality-assurance programmes require monitoring and recording of the procedures adopted for the cryopreservation of oocytes. These data may be used at later stages for various purposes, for example, assessments of efficacy and reproducibility. Each laboratory should have operative instructions describing the cryopreservation protocols and flow charts showing the path that oocytes follow from recovery to their use after cryopreservation. All aspects pertaining 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.

Possible Effect of Slow Freezing on Human Oocytes

Fully grown oocytes from antral follicles may be cryopreserved at the immature—germinal vesicle (GV)—or mature stage. Although immature oocyte cryopreservation has been recently suggested to represent a possible route for fertility preservation especially in women facing the prospect of gonadotoxic therapies [1], the storage of mature oocytes has gained a far larger interest as an alternative to embryo cryopreservation. The pre-ovulatory mature oocyte is an individual entity, ready for fertilization and relatively autonomous from cumulus cells which 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 contacts, 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 [2, 3], 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. Overt oocyte death may be observed immediately after thawing 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. Cell death may occur even hours after the completion of the process of thawing and rehydration. Gook et al. [4] 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, about 30 years ago, Sathananthan observed fractures in the zona pellucida, oolemma irregularities, and widespread disorganization of the ooplasm. After exposure to dimethyl sulfoxide (DMSO) at 0°C, he also found that parts of the endoplasmic reticulum, Golgi, mitochondria, and the cytosol were damaged to some

extent [5]. 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 [6] described cytokinesis anomalies and cleavage arrest in embryos developed from cryopreserved human oocytes, despite electron microscopy analysis carried out after thawing had not shown gross cytoplasmic alterations. Afterwards, 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 nineties to ensure high survival rates. With the development of improved protocols [3, 7, 8], ultrastructural studies again attracted a significant interest. By comparing fresh and frozen–thawed oocytes, Ghetler et al. [9] 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 it became available, ICSI was chosen as the elective route to achieve fertilization in cryopreserved oocytes [10] and is now recognized as a standard of treatments involving cryopreserved oocytes [11–14]. Despite that, the question of a non-physiological release of CG in cryopreserved oocytes remains open. By using epifluorescence microscopy, Gook et al. [4] 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 [15]. The possibility to use standard IVF has been reported by other authors [16]. 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 [17]. Similar findings were described by Gualtieri et al. [18]. Consistent with this, oocytes stored by a CRSC protocol including ethylene glycol (EG) as an intracellular CPA [19] or by vitrification [20] 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 cryopreserved 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, although the detection of thickening and change in texture of the inner surface of the ZP is in line with this hypothesis [17].

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 and have been interpreted as a sign of cytoplasmic immaturity or, vice versa, ageing. Because vacuoles may also derive from a general response to injury [21], 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 [9, 17, 19]. It is tempting to hypothesize that vacuoles located peripherally may evolve from crypt-like invaginations and clusters of endocytic vesicles which form in the oocyte cortex following simple exposure to CPA [22]. 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 [20]. Regardless 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 [17] and a reduced developmental ability [11, 23] are concomitantly associated to certain CRSC protocols, although this has not been confirmed through a prospective randomized control trial. Preliminary evidence suggest that vitrification protocols may be associated with high implantation ability [14] and a normal or low occurrence of vacuoles [20].

Mitochondria and elements of the smooth endoplasmic reticulum (SER) 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 in antioxidant defence. 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 which are triggered by the fertilizing spermatozoon and are interpreted by the oocyte biochemical machinery as a start signal for fertilization [24]. Magnitude and frequency of calcium oscillation have also been found to influence much later events of development, such as organogenesis and fetal growth [25]. For such reasons, mitochondria-SER aggregates are particularly important for the physiology of the oocyte. Mitochondria and SER elements, separately or 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 [17] and used in the clinical practice [11, 23]. This is a

reassuring evidence in the light of the role of mitochondria-SER associations in the mechanism of intracellular calcium signalling, 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 [19]. 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, mitochondria-SER aggregate disarrangements 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 [20]. However, such findings are largely insufficient to conclude that EG can specifically and directly affect the constitution of mitochondria-SER aggregates. Overall, these studies confirm the usefulness of TEM to investigate oocyte quality after cryopreservation.

One of the factors that could cause a partial loss of oocyte viability after cryopreservation is the phenomenon of in vitro ageing. 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 [26]. 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 [3], 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 [27]. 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 dynamics in frozen–thawed oocytes [28]. 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 human oocytes [29, 30].

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, constrains of religious, ethical, or legal nature limit 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 [11]. 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 has been adopted systematically and described in various studies conducted [12, 23, 29, 31]. 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 [32, 33]. The different implications of these two strategies are rather obvious. The thawing of only a few oocytes per cycle involves a higher risk of cycle drop out as an effect of high rates of fertilization or cleavage failure [11]. 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, affects 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 [11, 34]. An example of the inadequacy of pregnancy rate per transfer as a measure of efficacy is offered by a study [31] in which by using a freezing protocol specific from cleavage stage embryos, 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 [35, 36] have 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 at different stage of the process (fertilization, cleavage, selection for freezing, and thawing), they estimated that from an original pool of 100 fresh oocytes, about 5 implantations from frozen embryo can be achieved. Gook and Edgar suggests that the same kind of analysis 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 know that a considerable increase (35–40% to 70–75%) in the survival rate oocytes cryopreserved by CRSC may be obtained by augmenting 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 [12, 23, 31]. 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 [11, 31]. Therefore, in 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, implantation rates per thawed oocyte of about 5% can be achieved, a result that make oocyte freezing rather comparable to embryo freezing. By adopting a freezing protocol based on sodium-free media, Boldt et al. [33] reported an implantation rate per thawed oocyte 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 [2] 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). Parmegiani et al. [29] have also achieved a result of a similar magnitude. However, in general, these studies are rather numerically smaller in comparison to those involving frozen embryos. For example, Edgar et al. [37] reported an experience based on more than 5,000 frozen–thawed embryos.

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. [11] described 11 births from cryopreserved oocytes. These children had an average weight of 3.2 kg, normal karyotype, and no malformations. Shortly afterwards, Chen et al. [32] reported the birth of 5 children from cryopreserved oocytes with normal karyotypes. Other authors [2, 12, 23, 31, 33, 38, 39] 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. [12], a mean gestational age of 37.1 weeks and weight of 2,807 g were observed. So far, the largest group of children from cryopreserved oocytes has been described in a preliminary study of Borini et al. [40] where 4 cases of developmental anomalies were found (Rubinstein–Taybi syndrome, 47XXX, chromosome 19 hypomethylation, and choanal atresia) in 146 births. Tur Kaspa et al. [41] analysed 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 [42]. However, the absence of an association between oocyte cryopreservation and congenital anomalies is confirmed by a different analysis including over 900 births [43]. 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.

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 novel theoretical models which can predict better cryopreservation conditions and thereby improve oocyte quality after freezing–thawing.

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Vitrification: Research in Animal Models

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Gábor Vajta

Abstract

During the past 50 years, impressive success has been achieved in cryopreservation of male gametes and preimplantation stage embryos of mammals. Techniques were established mostly in domestic animals, predominantly in cattle, because of the high demand of the breeding industry. Artificial insemination with frozen-thawed semen in cattle is now the most frequently used assisted reproductive technique in mammals. However, the advancement in cryopreservation of female gametes was slow, and the efficiency is frustratingly low. The only exception is mouse, where traditional freezing of oocytes has resulted in high survival and developmental. Experiences obtained from oocyte cryopreservation of some domestic species provided much more important information for establishing a successful human oocyte cryopreservation method, although none of the tested domestic species have exactly the same oocyte type of cryosensitivity as humans. In spite of controversies and differences, research in domestic animals has contributed considerably in the establishment of a successful human oocyte cryopreservation system that is now acknowledged worldwide and may be regarded as the greatest achievement in reproductive cryobiology in the first decade of our century.

Keywords

Mammalian embryology • Mammalian oocyte • Cryopreservation • Cryoprotectants • Vitrification • Slow-rate freezing

Cryopreservation of Mammalian Oocytes: The Ultimate Challenge in Mammalian Cryobiology

During the past 50 years, impressive success has been achieved in cryopreservation of male gametes and preimplantation stage embryos of mammals. Techniques were established mostly in domestic animals, predominantly in cattle, because of the high demand of the breeding industry. Artificial insemination with frozen-thawed semen in cattle is now the most frequently used assisted reproductive technique in mammals. Transfer of frozen-thawed morulae and blastocysts is also a routine technology, although the numbers of transferred embryos are stagnating worldwide since years for reasons not closely related to the efficiency of the technology. To a lesser extent, the same techniques are also applied in sheep, goats, horses, and pigs. These techniques are also successful in experimental animals, especially in the most widely used species, mouse.

However, the advancement in cryopreservation of female gametes was slow, and the efficiency was—and still is—frustratingly low. The only exception is mouse, where traditional freezing of oocytes has resulted in high survival and developmental rates [1]. Curiously, this fact had a

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controversial effect on the development of a successful cryopreservation technique for humans, as methods successfully used in mouse were inappropriate for human oocyte cryopreservation. In this regard, experiences obtained from oocyte cryopreservation of some domestic species provided much more important information for establishing a successful human oocyte cryopreservation method, although none of the tested domestic species have exactly the same oocyte type of cryosensitivity as humans.

In general, the problems related to oocyte cryopreservation are attributed to several physical and biological factors [2] including the size and shape of oocytes. The extremely large size—oocytes are close to be detectable with naked eyes—and almost perfect round shape make equal distribution of potentially harmful cryoprotectant solutions within the cells difficult, accordingly toxic damage may develop at the periphery while the center is still not protected by the cryoprotectants. Additionally, oocytes in most mammalian species including humans are very sensitive to chilling injuries, that is, damages between +15 and −5°C. This sensitivity is generally attributed to the injury of the meiotic spindle, lipid-containing membranes, and lipid droplets. The damage of the spindle may be reversible; however, the injury of lipid structures, especially the lipid droplets, seems to be fatal, although the specific function of these droplets is still unclear [3–7]. The premature release of cortical granules caused by the cryopreservation process may also cause zona hardening and hampering sperm penetration.

On the other hand, parallel investigations with other developmental stages suggest the role of other, still unknown factors contributing in this sensitivity, as zygotes with the same size are much more tolerant toward cryoinjuries; the absence of the meiotic spindle of immature oocytes would suggest less cryosensitivity, but in fact, the contrary is true; and data regarding cryopreservation-induced cortical granule exocytosis and zona hardening are controversial; besides, this problem can be easily eliminated by the application of intracytoplasmic sperm injection. Moreover, oocytes of different mammalian species seem to suffer different types of injuries, for example, morphological survival of bovine oocytes is easy to achieve, but the seemingly intact oocytes may fail to get fertilized and activated, while in humans, the signs of cryodamage are easy to recognize minutes after warming; on the other hand, a light microscopically intact MII phase human oocyte after warming has almost or entirely the same developmental competence as its noncryopreserved counterpart.

In spite of these controversies and differences, research in domestic animals has contributed considerably in the establishment of a successful human oocyte cryopreservation system that is now acknowledged worldwide and may be regarded as the greatest achievement in reproductive cryobiology in the first decade of our century.

Research in Domestic Animals Promoting Human Embryology

As mentioned above, the initial embryo cryopreservation techniques based on traditional freezing were successfully adopted for mouse oocytes, but—apart from some exceptional achievements—the human application resulted in very low efficiency that hampered to achieve any practical goals. Accordingly, embryologists had to follow to ways for improvement: to find an entirely new approach or to make stepwise efforts to increase the efficiency of traditional freezing. During the past 2 decades, serious efforts were made in both direction, and eventually, the outcome of both ways has justified the invested energy. Experiments in domestic animals contributed mostly in the former way—to find a radically different solution for the problem—and the latest comparative experiments seem to prove the superiority of this approach.

Except for some sporadic reports in several species, traditional freezing of oocytes of domestic animals was proved to be a dead end. Accordingly, the alternative of traditional slow-rate freezing, vitrification, was applied for the purpose.

Unfortunately, oocyte cryopreservation did not and still does not belong to the priorities in domestic animal embryology. This research area is almost entirely motivated by commercial factors, to increase quantity and quality of livestock. While sperm and embryo preservation is a strategic goal, only very few reasons and very special situations justify the cryostorage of oocytes. Although the benefits of conservation of maternal genome may be considerable in some elite animals, or endangered breed, in practice, this possibility is almost entirely disregarded. Accordingly, achievements in this field were the results of enthusiasm of a few devoted groups, and their efforts were never rewarded. In contrast, research in this direction was financially unsupported, rarely tolerated, and most of offspring derived from cryopreserved oocytes as the result of painstakingly long efforts ended up with early slaughter. Paradoxically, this very limited and unsupported research has eventually led to the establishment of the most successful technology of human oocyte cryopreservation.

General Features of Techniques Used for Oocyte Cryopreservation

All cryopreservation methods attempt to reduce damages during the procedure and—to a lesser extent—to support the regeneration of biological samples from the unavoidable injuries. Most damages develop during the cooling and warming process—some of them at seemingly harmless high temperatures above or around 0°C, while the storage itself below −150°C, most commonly at −196°C, is a relatively

harmless part of the procedure. The commonly accepted two major cryopreservation techniques that have emerged over the past decades for oocytes and embryos use different approaches to achieve these goals. Traditional slow-rate freezing attempts to establish a balance between various sources of injuries (ice crystal formation, toxic injury, osmotic and fracture damage, etc. [8]; reviewed in detail recently [9, 10]) by minimizing all of them to a tolerable level. As indicated by the name, the cooling rate is relatively slow; accordingly, samples are held for a relatively long period of time under physiological temperature close to 0°C and accordingly may suffer chilling injury [7, 11–13].

The principle of vitrification is the total elimination of ice crystal formation. In practical work, there are several ways to achieve this goal: to increase the concentration of cryoprotectants, to increase both cooling and warming rates, and to minimize the volume of the solution containing the sample to reduce the chance of ice nucleation. The initial vitrification methods were based on the traditional tools of cryobiology (standard insemination straws or cryovials); accordingly, the achievable cooling and warming rates were limited, and the benefits compared to traditional slow-rate freezing were moderate. Later, special tools were applied or developed for the purpose including electron microscopic grids, narrow plastic or glass capillaries, loops, and thin plastic filmstrips (reviewed by Vajta et al. [14]), allowing the increase of cooling and warming rates with an order of magnitude. This approach offered two benefits: a considerable reduction in the required concentration of cryoprotectants and the dramatic decrease in chilling damage, as samples passed extremely quickly through the dangerous temperature zones (+15 to −5°C) and reached the safe higher or lower degrees at cooling and warming, respectively; accordingly, the time was restricted for the damage to develop in full extent. This benefit which was initially regarded as a by-product of new vitrification techniques has eventually become the major advantage of the application of vitrification for chilling sensitive samples including the MII phase mammalian oocytes [15, 16].

Unfortunately, the required high cooling and warming rates require a direct contact with the cooling and warming solution, that is, with liquid nitrogen or the rehydrating solution containing the osmotic buffer. This direct contact may result in liquid nitrogen-mediated cross-contamination and disease transmission. Although cryopreservation of samples in mammalian embryology was not proven to be responsible for any disease transmission, the theoretical danger exists, and the possibility has also been proven under experimental conditions [17]. There are several ways to eliminate the possibility of contamination: performing cooling and warming in sterile solutions while storing samples in safe containers or in the vapor of liquid nitrogen, using sterile metal surfaces for cooling, or applying sophisticated closed systems [18–22]. However, in some of these systems, either the overall efficiency seems to be compromised or the security or practicality of the system is compromised. Accordingly, the ultimate solution for establishment of a fully safe and highly efficient vitrification system still requires future research.

Results Achieved in Animal Models

As mentioned earlier, the first success in mammalian oocyte cryopreservation has been achieved in mouse by traditional freezing [1]. Even the birth of the first human baby preceded by 6 years the first achievements in domestic animals ([23, 24], respectively). However, vitrification has become soon the preferred approach for cryopreservation of mammalian oocytes. The first mouse pups achieved from vitrified oocytes were reported by Nakagata in 1989 [25]; and in 1992, in parallel with traditional freezing, vitrification has also resulted in the birth of a healthy calf [26]. During the subsequent years, vitrification has become the choice when cryopreservation of domestic animal oocytes was considered.

The introduction of special tools and high-rate cooling and warming techniques had significant impact on the efficiency [14, 27]. Refinement of these techniques has also permitted the birth of calves after vitrification of immature oocytes [28], even with a subsequent round of blastocyst vitrification [29]. Cattle cytoplasts (enucleated oocytes) were also successfully used after vitrification for embryonic or somatic cell nuclear transfer [30, 31].

The extreme chilling sensitivity—due to the high cytoplasmic lipid content—has hampered cryopreservation of porcine embryos and oocytes for long. Eventually, physical removal of these lipid droplets [32] has improved outcomes, but the required micromanipulation has made the procedure too complicated for routine application. A modification of this technique including partial zona digestion and high-speed centrifugation to separate lipids from the remaining components of the cytoplasm [33] opened the way for obtaining live piglets after somatic cell nuclear transfer by using either zona-included or zona-free oocytes [34, 35].

In other domestic species, only sporadic attempts were reported, including horse [36] and buffalo [37]. No reports are available about full-term development in sheep and goat after oocyte cryopreservation, although late-stage fetuses have been achieved (Ledda, personal communication).

Future Perspectives

Although a very limited number of offspring were born after oocyte cryopreservation in domestic animals, the achievements have paved the road for establishment of a highly successful method in humans. Accordingly, innovative

approaches applied in domestic species may open new possibilities for future human application, too.

One possibility is the application of cytoskeleton relaxants. The initial approach with cytochalasin B was reported to be successful in pigs [38–40]; however, the benefits were questioned by others [20, 41]. Results achieved with Taxol seem to be more equivocal and promising [40, 42–45], but the human use of this potent anticancer agent to promote cryosurvival of healthy oocytes may not be justified [46].

The other, more recent possibility to improve cryosurvival of oocytes is the pretreatment with carefully selected and strictly controlled sublethal stress effect to increase the stress tolerance. The first and so far the most significant approach toward this direction was the application of high hydrostatic pressure (HHP). In porcine oocytes, the treatment does not result in light microscopic alterations, but induced higher resistance and developmental competence after various interventions including cryopreservation [47, 48]. As an analogue, osmotic pressure was also applied successfully before cryopreservation to increase survival and developmental rates [49, 50]. These achievements may only mean the start of a promising new approach, which may eventually help to increase the efficiency of cryopreservation of oocytes in many other mammalian species including humans.

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Oocyte Vitrification

57

Ana Cobo

Abstract

Oocyte cryopreservation represents a great alternative for many women, becoming a promising choice especially in cases of patients suffering from cancer malignancy. In these women, the option to reliably cryopreserve oocytes and store them with the intention to safeguarding their fertility would be quite welcome. In addition, oocyte cryopreservation is the most valuable option for women who wish to delay their motherhood, a choice that is increasingly frequent, especially in developed countries, raising the proportion of women at advanced ages that desire to get pregnant. Oocyte cryostorage results 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, the most important, the absence of a quarantine period. Oocyte cryostorage brings additional advantages to ART programs being helpful to solve different clinical situations as in low-responder patients, unpredictable unavailability of semen sample collection from the male partner, risk of suffering ovarian hyperstimulation syndrome, or some other cases in which the embryo transfer is not advisable.

Keywords

Oocyte cryopreservation • Oocyte vitrification • Cryostorage of oocytes • Fertility in cancer malignancy • Cryostorage of oocytes • Vitrification of oocytes

The essential role of cryopreservation in ART has become obvious since the commencement of the infertility treatment, becoming a more flexible and efficient practice. Semen and embryo cryopreservation has been a successful strategy, routinely applied in IVF procedure for a long time. However, in spite of numerous studies conducted over the last 20 years, the reliability of oocyte cryopreservation is just being confirmed currently. All of the efforts that have been made are clearly justified, mostly because an efficient oocyte cryopreservation program would be quite welcome and widely applied in ART, since there are many other indications for oocytes cryopreservation different of the abovementioned egg banking for ovum donation. Potential beneficiaries of this technology would be cancer patients who need an option for fertility preservation before undergoing the potential sterilizing 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 pick up, due to a variety of reasons [5, 6].

Despite all of this wide diversity of potential applications, egg banking has not been a routine procedure until very recently; in fact, there are still very few centers worldwide that routinely apply oocyte cryopreservation in their clinical routine. This fact can be explained because the methodology to cryopreserve human oocytes has been disappointing, with results that have not always been reproducible. Lately, vitrification has proven to be a very efficient method for successful

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oocyte cryostorage. Some fundamental principles of cryobiology would be helpful to understand why it has been extremely difficult to reach the goal of safely cryopreserved human oocytes.

Cryobiology Background

There are some reasons that could explain the low rate of successes that has been traditionally observed. Some of them include the size, shape, and cell number regarding oocytes. These gametes are the largest cells of the human body, and this 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). Finally, considering the oocytes as a unique cell, they have only one chance of success, and there are few possibilities to restore from a serious damage; this situation is completely different from the one observed in tissues composed by millions of cells, 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 cooling 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, from +15 to −5°C 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 use of high cooling rates. Moreover, as the sample vitrifies, ice crystal formation is avoided [12].

Vitrification, in particular by means of methodologies that use a minimum volume, is altering this situation by producing results that have not been achieved with other approaches [5]. 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 high cooling rates from −15,000 to −30,000°C per minute, therefore avoiding the risk of chilling injury [12]. This ice-free cryopreservation method has undergone modifications in order to optimize results. One such modification has been to reduce the volume of the vitrification solution containing oocytes, which allows the CPA concentration and, consequently, the cytotoxicity to be decreased [13, 14]. As abovementioned, this procedure circumvents the two major limiting factors for achieving optimal cryopreservation: chilling injury [13] and ice formation [15]. Chilling injury can be minimized during vitrification by use of high cooling rates [12]. 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. To avoid ice formation, the vitrification technique makes 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 CPAs [5]. In this way, a combination of two or three of these agents can decrease the individual specific toxicity. The most common mixture employed for this purpose consists of EG, DMSO, and sucrose [5]. To optimize the results, in addition to an appropriate selection of CPAs, it is also helpful to use these agents at lower concentration while maintaining the necessary concentration 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 CPA. Several approaches fit these conditions. The “minimum drop vitrification” method was proposed by Arav, using a very small volume of vitrification solution placing the samples on a specific device which must be cooled very quickly [17]. High cooling rates are achieved when samples are loaded in minimum volume that are directly immersed in LN. These methods are also known as open systems. Such high rates are impossible to achieve with hermetically closed vials, which are used in closed systems. 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 has been considered as a potential source of pathogen agents. Even though, no any case of cross-contamination has ever been reported in ART in many years of practice, highlighting that such eventuality could be extremely unlikely.

On the other hand, the limit imposed by the cooling rate could be restricted to oocytes since satisfactory results have been obtained after the vitrification of embryos at different developmental stages using closed systems. In any case, there are no formal comparative studies which make possible to draw definitive conclusions. A wide variety of open approaches have been reported in the literature [18–23] as well as closed ones [24, 25].

Clinical Outcomes

A review on human oocyte cryostorage based on all reports on vitrification MII oocytes and providing data regarding implantation and pregnancy outcome depicts the situation of the clinical application of human oocyte cryopreservation [26]. It is worth mentioning that nearly all of these studies assessed employed open systems for vitrification, highlighting the advantage of these systems with regard to oocyte cryopreservation and its most wide application for clinical practice in humans. Within this session, we will focus on our clinical experience using the Cryotop method for vitrification [27]. This device consists of a fine transparent polypropylene film attached to a plastic handle and equipped with a cover straw, into which can be loaded very small volumes of oocytes (up to ~0.1 μL), 10 times lower than the capacity of other minimum volume devices [27].

Our first approach with the vitrification of donor oocytes using the Cryotop method was carried out through the simultaneous comparison of the outcome of both vitrified and fresh oocytes from the same ovarian stimulated cycle; we confirmed the potential of vitrification of oocytes using this methodology [28]. We obtained a 96.7% survival rate and detected no significant difference in fertilization rates (76.3 and 82.2%), day 2 (94.2 and 97.8%) or day 3 embryo cleavage rates (77.6 and 84.6%), or blastocyst formation rates (48.7 and 47.5%) for vitrified and fresh oocytes, respectively. In that study, either vitrified or fresh oocytes belonging to the same cohort were simultaneously inseminated using the same semen sample. In this way, we were able to analyze the potential of vitrified and fresh oocytes under exactly the same conditions. The ratios of good quality embryos on day 3 and at blastocyst stage were similar in both groups. Pregnancy, implantation, miscarriage, and ongoing pregnancy rates (OPRs) per transfer were 65.2, 40.8, 20, and 47.8%, respectively. The potential use of this approach for fertility preservation in cancer patients as well as in other clinical situation in ART has also been highlighted [1, 29].

Although these evidences have been encouraging, the need of a controlled clinical trial to test the efficiency of oocyte vitrification was mandatory. A randomized, prospective, triple-blind, single-center, parallel group controlled clinical trial, including 600 recipients from our ovum donation program, aimed to compare the outcome of vitrified-banked oocytes to the gold standard procedure employing fresh oocytes that has been published by our group [30]. The study was designed to establish the superiority of the OPR of fresh oocytes over that of vitrified oocytes. The primary end point, the OPR per intention-to-treat (ITT) population, was 43.7% for vitrified oocytes, over 41.7% for fresh ones. As shown by the OR, we failed to prove the superiority of fresh oocytes over the vitrified oocytes, and moreover, we were able to assume the noninferiority of the vitrification group. Additionally, the proportion of top-quality embryos obtained either by inseminated oocyte (30.8% vs. 30.8% for day 2 and 36.1% vs. 37.7% for day 3, for vitrified and fresh oocytes, respectively) or by cleaved embryos (43.6% vs. 43.8% for day 2 and 58.4% vs. 60.7% for day 3, respectively) was similar between groups.

The outcome achieved in this controlled randomized clinical trial confirmed demonstrates that cryobanking by applying the vitrification technology can provide successful clinical outcome in oocyte donation programs helping to the validation of this strategy. With no doubt, it is of great importance to ovum donation procedures, as it allows traditional drawbacks associated with the use of fresh oocytes to be overcome. Supported by all this evidence, we have established an oocyte bank that currently operates to meet the basic needs of our donation program contributing greatly to improve the logistics of the program and, what is also relevant, allowing us to keep the quarantine period. To date, we have performed 1,856 ovum donation cycles involving 23,000 cryostored oocytes with an overall survival rate over 90%. A mean number of 1.6 ± 0.7 embryos have been replaced, and an OPR of 47.7% has been achieved. A 63.6% of the patients were able to vitrify surplus embryos; therefore, the cumulative OPR after cryotransfers has been over 60%. Additionally to ovum donation, we have applied oocyte cryopreservation in cases of infertile patients with own oocytes due to the risk of suffering ovarian hyperstimulation syndrome (OHSS), no available semen sample the day of ovum pick-up or many other situations in which the embryo transfer was not advisable. In nearly 450 cycles (mean age 47.4 ± 4.5), the overall survival rate was 84% with a clinical pregnancy rate of 41.5% and 32.8% of implantation rate. Oocyte vitrification has been applied for fertility preservation in cancer patients ($N = 70$; mean age 32.7 ± 5.7) and for social reasons ($N = 158$; 35.2 ± 5.8), however no warming procedures have been performed to date.

Other authors have confirmed the excellent profile of oocyte vitrification either with autologous or donated oocytes. Rienzi et al. showed in a prospective, randomized sibling study conducted with autologous oocytes that oocyte vitrification procedure is not inferior to fresh insemination procedure achieving comparable ongoing clinical pregnancy rates [31]. A very recent study has prospectively compared an open vs. closed system between fresh and sibling vitrified oocytes [32]. These authors have observed an impairment of embryo quality after employing the closed system. In contrast, the parameters analyzed were similar between fresh and sibling oocytes vitrified using the open system. Other authors have also endorsed the suitability of oocyte vitrification by the Cryotop method in an oocyte donation program [33]. These authors recommend this approach to be applied in infertile patients with different indications within a routine IVF program. All these evidences strongly support the efficiency of oocyte vitrification.

Practical Aspects of Oocyte Vitrification

Laboratory Procedure

- Aspirate the oocytes from the culture dish and keep them at the tip of the capillary or Pasteur pipette and transfer them within minimum drop volume to 20 μL of buffer solution (BS) supplemented with 20% serum substitute into a reproplate well (Kitazato, Biomedical Supply, Tokyo, Japan) (Fig. 57.1).
- Add 20 μL of equilibration solution (ES) containing 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO) surrounding the previous drop containing the oocytes (Kitazato, Biomedical Supply, Tokyo, Japan). Wait for 3 min at room temperature (Fig. 57.1).
- Add another drop of 20 μL of ES in the same way as in the previous step and wait for 3 min more.
- 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 (Fig. 57.1).

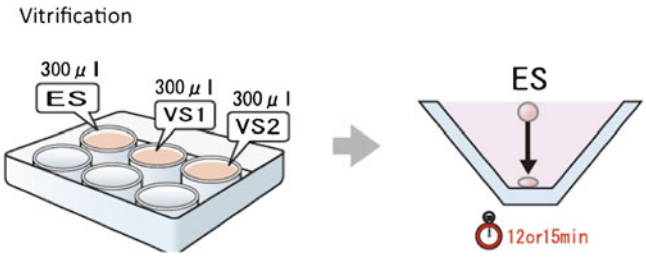

Fig. 57.1 Equilibration procedure

- After equilibrium, aspirate the oocytes at the tip of the pipette. Afterward, place them on the surface of the vitrification solution (VS) containing 15% EG plus 15% DMSO in BS solution (Fig. 57.1). Try to carry a minimum volume of ES solution. Note that the oocytes will float due to the high density of the VS media.
- Dispense 300 μL of VS solution into two well of the reproplate (Kitazato, Medical Supply, Tokyo Japan). 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 (Fig. 57.2).
- 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).
- 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.
- Aspirate the oocytes and maintain them at the tip of the pipette within minimum volume of VS.
- Place the Cryotop under the microscope.
- Proceed to load the oocytes onto the Cryotop within minimum volume.
- Aspirate excess medium to make sure they are contained within minimum volume.
- Immerse the Cryotop directly into the liquid nitrogen container (Fig. 57.2).
- Place the plastic protector Cryotop.
- Transfer the oocytes to the storage tank.

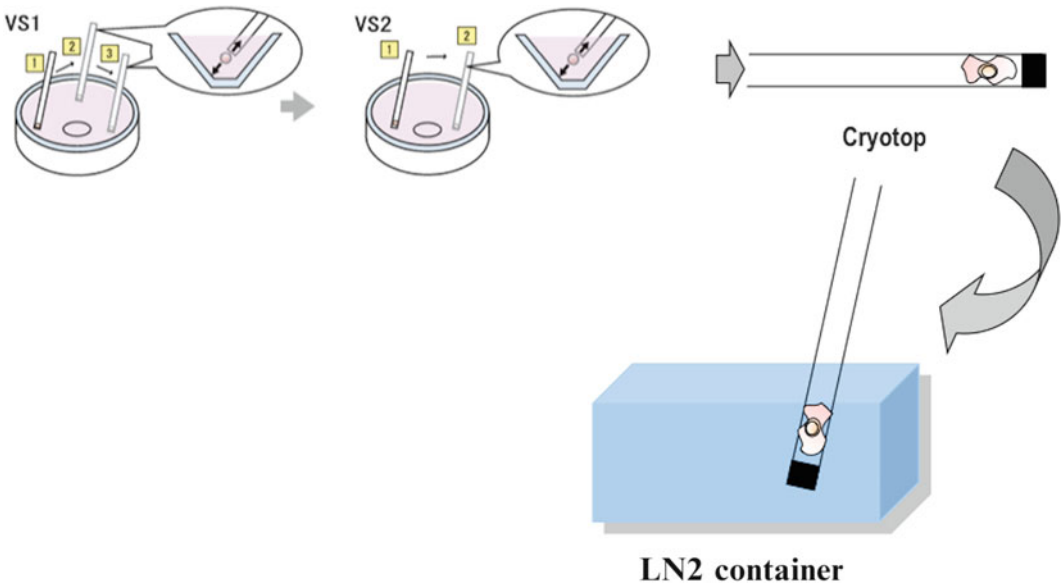

Fig. 57.2 Vitrification procedure

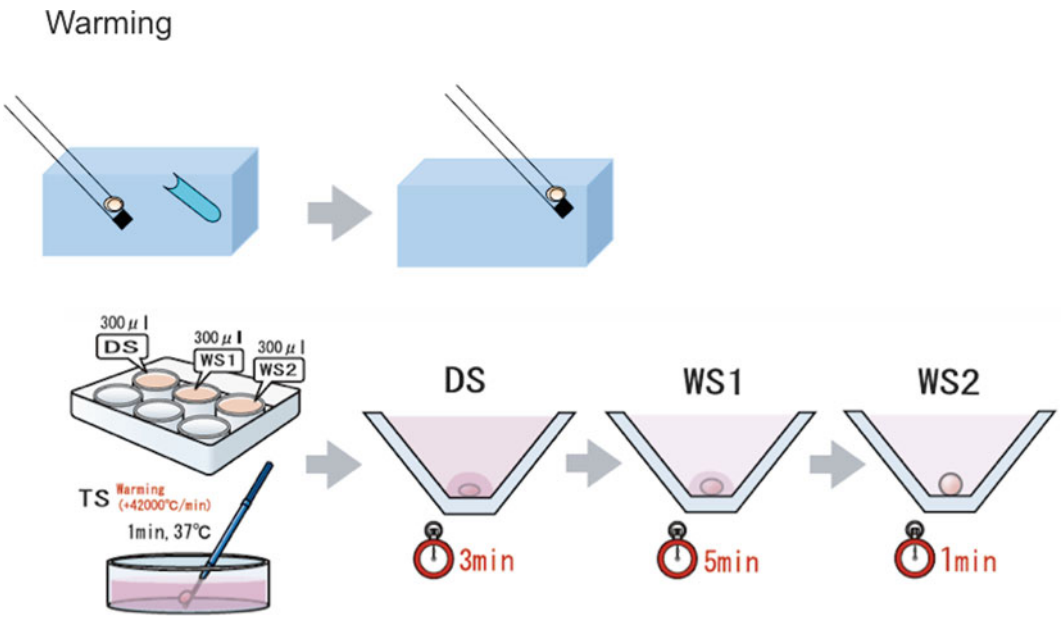

Fig. 57.3 Warming and dilution procedure

Warming Procedure

- Remove the Cryotops from the storage tank and handle them submerged into liquid nitrogen. Avoid temperature changes.
- Remove the protective straw maintaining the Cryotops immersed in liquid nitrogen.
- Remove Cryotop from the liquid nitrogen container and submerge it instantly (straight and quick movement) into 1.5 mL of a solution containing 1.0 M sucrose (TS) (Kitazato, Medical Supply, Tokyo, Japan) (Fig. 57.3).
- Visualize the oocytes and immediately start the countdown to 60 s.
- Do not manipulate the oocytes within the first 40 s.
- Retrieve the oocytes from the Cryotop with very gentle manipulations within a minute in TS solution.
- Transfer the oocytes to the DS well in the reproplate (Fig. 57.3). Aspirate the oocytes very gently and continue to aspirate TS until the column reaches approximately 2 mm in length (Fig. 57.3).
- Take the pipette to the DS well and immersed 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 (Fig. 57.3). Leave the oocytes on the "top" of this column. Wait for 3 min.
- Transfer the oocytes to well WS (BS supplemented with 20% SSS) exactly as in the previous step. Wait for 5 min.
- 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.
- Transfer the oocytes to the regular culture dish and place them in the incubator at 37°C Wait for 2 h before ICSI.

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Slow Freezing of Embryos

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Abstract

Cryopreservation of human embryos was introduced more than 2 decades ago, mainly as an approach to save supernumerary embryos produced after ovarian hyperstimulation. The Society for Assisted Reproduction Technology and the European Society of Human Reproduction and Embryology both continuously publish statistics on embryo freezing and birth rates in the United States and Europe, respectively. These are valuable data 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. 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. A further driving force for an increased used of vitrification is the practicality of the technique. Apart from being much more cost effective, the procedure requires significantly less time when compared to the long equilibration methods required by typical slow-freezing protocols. Despite these clear advantages of vitrification, many labs still use slow freezing, mainly because of the wealth of clinical data that supports the technique. Switching a well-established, effective method for a new protocol also seems intimidating, thereby preventing implementation of newer technologies. We predict that, in the future, vitrification may become the sole method used for clinical embryo cryopreservation.

Keywords

Embryo cryopreservation • Cryobiology • Infertility • Slow freezing • Cryoprotectants • Cryodamage • Freeze tolerance • Vitrification

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 have the ability to 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, and the large number of

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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 lead to the successes that were ultimately reported during the first half of the twentieth century. Here follows a condensed representation of the history of modern cryopreservation, but a more detailed review can be found in ref. [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 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 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 the 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 it 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 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 2 M sucrose solution. Although about 40% initially lost their motility during incubation in the hypertonic solution, all the 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 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 embryos. 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 [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 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 ref. [12].

During the late 1950s and early 1960s, Peter Mazur turned his attention toward elucidating mechanisms involved in cell damage during freezing and thawing. Although these funda-

mental principles will be discussed in the following section, it is noteworthy that his discoveries 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. This was followed by more successes in other mammalian species [14, 15], and finally human embryos in the early 1980s [16]. Almost 30 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. 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.

Mechanisms of Freeze Damage

Cells are composed 60–85% of 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 (for review, see ref. [17]). 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 occurs 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 [17].

Another occurrence during osmotic shrinkage is hydrostatic pressure that 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.

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. This can be detrimental, since sharp 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 allow complete equilibration of the extracellular solution 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 [18].

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 ref. [19].

Thawing of cells present 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 [17]. 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. One conclusion that is clear from published works is 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 which warming method will lead to the highest post-thaw survival rates.

Components of Slow Embryo Freezing

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 normally used in combination to dehydrate (non-permeable) and equilibrate (permeable) cells prior to freezing. Their rate of diffusion into the cell is slower than that of water exiting through osmosis, which necessitates some time for equilibration. 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 these agents 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 [20]. 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 ref. [21].

Media Formulations and Procedures

As discussed above, numerous different cryoprotectants can assist in protecting embryos from 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.

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 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 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 standard procedure to included two small air bubbles before and 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 are 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 supercooling. 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 recrystallization may occur in the small time window during which the liquid will transition trough 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.

Factors That Determine Success

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 affect of surface-volume ration on cryopreservation success (for review, see ref. [17]), 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 [22–24]. 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 [25]. 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 (days 2 and 3)-stage frozen-thawed embryos were compared [23, 26]. 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.

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 [22–24]. One major advantage is that the possibility of future embryo biopsy is still 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 what is the case with more advanced embryos since the only measurable quality parameter at this stage is the presence or absence of pronuclei—and zygote scoring may not be as predictable as cleavage- or blastocyst-stage assessment. After thawing, pronuclear stage embryos are typically cultured for at least 1 day—but many times, more days 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.

Cleavage Stage

The majority of embryo cryopreservation over the past 2 decades was performed using propanediol and sucrose to slow freeze at the cleavage stage [27]. Survival rates of 60–88% were reported [28–30]. 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. Furthermore, it preserves the opportunity to do a day 3 biopsy for genetic screening.

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 perivitelline space [31–33]. The reason for the neglect is not due to poor results, but rather 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. However, if a patient has too few embryos or they are of suboptimal quality, day 3 transfers are 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.

Blastocyst Stage

Although the early successes in embryo cryopreservation were achieved at the blastocyst stage [34, 35], embryo freezing at this advanced stage was not routinely used until recent years when blastocyst culture systems became optimized. Culturing embryo 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 [25]. 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 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 be removed 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.

Embryo Quality

Several studies showed 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 [36–38]. A similar observation was made in the bovine model, where blastocysts of high quality survived freezing better than those of lower quality [39]. Interestingly, Check et al. [40] reported that 4-cell embryos had a similar freezing tolerance to 6–8-cell embryos, suggesting that a slower developmental rate is not included in the quality parameters that affect freeze survival [39]. 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.

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 LCR, during which the cryodamaged blastomeres are aspirated from the embryo. 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" [41]. 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 [42] and promote frozen-thawed embryonic development [43]. LCR has also been used following human embryo cryopreservation at multicellular stages, and results showed that it could significantly improve clinical outcomes [44–46]. It should be noted that in the mentioned experiments, assisted hatching was performed in conjunction with LCR, a practice that was also shown beneficial after vitrification and warming [47] 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.

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 [48]). Various effective techniques have been described, including acid Tyrode's solution drilling/thinning [49], mechanical partial zona dissection [50], laser drilling [51], piezo drilling [52], enzymatic zona digestion [53], and most recently, mechanical zona expansion through hydrostatic pressure [54]. 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 [55, 56]. 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 [57, 58]. Therefore, it is 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.

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 step-wise 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 [59]. However, when patients order genetic screening, embryologist may have no choice other than 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.

Applications and Impact

Cryopreservation of human embryos was introduced more than 2 decades ago, mainly as an approach to save supernumerary embryos produced after ovarian hyperstimulation [16, 34, 60]. 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 United States and Europe, respectively. These are valuable data 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. Since vitrification is becoming more widely used, the weight that slow freezing has in the overall rates is likely decreasing significantly each year, as more clinics are switching over to vitrification for reasons discussed below.

ESHRE reported almost 460,000 in vitro cycles in 2006, of which more than 86,000 (15%) were frozen ETs [61]. Data from SART indicated over 140,000 cycles in the United States, of which about 21,400 (19%) were frozen-thawed ETs. As clinics and patients become more comfortable with the idea of embryo cryopreservation, these percentages should steadily increase. Since we know that at the moment, the large majority of clinics currently still use slow freezing, the impact of this technology on infertility treatment is obvious.

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 increase 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 (sET) 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 now limits the number of embryos that can be transferred. Embryo freezing 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. In Germany, for example, it is illegal to culture more than three embryos from one cycle at a time. Freezing extras 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.

Another reason why patients may want to freeze embryos is in cases where their ET is canceled due to hyperstimulation. Historically, before freezing became available, this condition required cancellation of the entire treatment cycle and loss of the embryos. Now, with embryo freezing 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.

Patients are becoming more interested in genetic screening of embryos. Although day 3 biopsies can be performed for FISH or array CGH analysis to give results in time for a day 5 fresh replacement, the increasing preference is to perform biopsy of blastocysts stage. Without the ability to cryopreserve the biopsied embryos while awaiting results, this would not be possible.

Some patients may have embryos left over after delivering the desired number of babies. Many patients have ethical problems with discarding embryos. A good option for them would be frozen embryo donation, thereby helping couples that could not conceive naturally or by in vitro techniques, and at the same time eliminate the financial burden of storing embryos without feeling guilty. Since the embryos can be thawed out any time, the recipient can be stimulated and scheduled for the ET whenever she is ready.

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 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 open vitrification techniques. Slow freezing is typically performed in sealed straws, ampoules, or cryovials, which all presumably prevent the 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). 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 is cumbersome. There are a few companies that are marketing closed-system vitrification equipment, but in our experience success rates are not comparable to that of open vitrification procedures. The caveat of the 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.

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 [62, 63]. A further driving force for an increased used 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 20 min versus approximately 45 min for slow-frozen embryos.

Despite these clear advantages of vitrification, many labs still use slow freezing, mainly because of the wealth of clinical data that supports the technique. Switching a well-established, effective method for a new protocol also seems intimidating, thereby preventing implementation of newer technologies. Although currently the vast majority of programs still utilize the slow-freezing method, the number of labs that uses vitrification increases every year and will likely continue to increase. We predict that, in the future, vitrification may become the sole method used for clinical embryo cryopreservation.

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 consult 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.

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 of a human embryo was less than 30 years ago [16]. Up till now, slow freezing has played the most prominent role. 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 will be completely replaced by vitrification. 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.

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Vitrification of Embryos

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Abstract

Vitrification is a very promising cryopreservation method with many advantages and an ever increasing clinical track record. There exist many variables that can profoundly influence the effectiveness and the survival rates of vitrified cells. A standardized vitrification protocol applicable to all stages of the preimplantation embryo may not be realistic because of (a) different surface-to-volume ratios; (b) differing cooling rate requirements between oocytes, zygotes, cleavage stage embryos, and blastocysts; and (c) 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.

Keywords

Vitrification of embryos • Cryopreservation • Vitrification protocol

Why Cryopreserve Embryos?

Successful cryopreservation of gametes in animal reproduction has a track record of over 60 years. By drawing upon the vast amount of knowledge on gamete and embryo cryopreservation accrued over the years, this led directly to the first pregnancy in humans derived from frozen-thawed embryos in 1983/1984. Since that time, the impact of cryopreservation on the growth and improved efficiency of assisted reproduction in humans has become increasingly appreciated. With approximately one quarter of a million babies born following cryopreservation, this technology has become a well-established and widely used routine procedure that allows important expansion of therapeutic strategies when IVF is used to treat infertility. Currently, controlled ovarian hyperstimulation protocols commonly provide embryos in excess of those needed for fresh transfer. Therefore, techniques have been developed to store these surplus embryos in liquid nitrogen (referred to as cryopreservation) for an indefinite period of time without significant compromise of their quality. Based on data from the Centers for Disease Control and Prevention (CDC) from 2001 to 2004, about 18% of all IVF cycles in the USA used frozen embryos for transfer. In addition, data from the same registry compared live births per transfer using frozen and fresh embryos (25% versus 34%, respectively), clearly showing that cryopreservation is an important adjunct to maximize the efficiency of every single patient's oocyte retrieval. The fundamental objectives for successful cryostorage of

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cells in liquid nitrogen at −196°C can be summarized as follows:

1. Arrest of cellular metabolism with maintenance of structural and genetic integrity.
2. Achieve acceptable cell survival post thaw/warming.
3. Protocol/technique must be consistent and reliable.

Furthermore, all methods and protocols for cryopreservation should be developed such that ice crystal formation and growth inside the cells or tissues must either be eliminated or massively suppressed. One recent hotly debated topic in the area of reproductive cryobiology is whether slow-cooling or rapid-cooling protocols both satisfy the fundamental cryobiological principles for reduction of damage by ice crystal formation during cooling and warming, and which approach is better. It is the case, nonetheless, that both methods of cryopreservation of biological material include six steps:

1. Initial exposure to cryoprotectant
2. Cooling (slow/rapid) to subzero temperatures
3. Low temperature storage
4. Thawing/warming
5. Dilution and removal of the cryoprotective agent
6. Return to a physiological environment

Although initially reported in 1985 as a successful cryopreservation approach for mouse embryos, vitrification has taken a backseat in human assisted reproduction. However, the practical advantages of this cryopreservation method have more recently caught the attention of many ART laboratories as a feasible alternative to traditional slow-freezing methods. Since 1985, 1,500 publications can be found referring to the topic of "vitrification," which is further evidence of the burgeoning growth of interest in this cryopreservation technology. One "drawback" considered by embryologists who are not familiar with the vitrification technique is the use of higher concentration of cryoprotectants, which does potentially mean that the vitrification solutions are more toxic than their counterpart solutions used for conventional slow freezing. However, with better understanding of the physical and biological principles of vitrification, this has led to numerous successful clinical applications of this technique within the field of assisted reproduction. As of today, all developmental stages of human embryos cultured in vitro have been successfully vitrified and warmed, with resulting offspring. Cryopreservation is one of the keystones in the overall picture of comprehensive clinical infertility treatment. These different embryonic stages will continue to be cryopreserved for a variety of different reasons whether practical, political, or personal. Today, slow-freezing technology still has the longest clinical track record and greater "comfort level" among embryologists. Nevertheless, vitrification with its increasing clinical application is showing a trend of greater consistency and better outcomes when compared to slow-freezing technology. Therefore, when (not if) IVF programs overcome the fear of the "unknown," and take on the challenge of the short learning curve with vitrification, then at that point, vitrification will become the clinical standard for human embryo cryopreservation.

Cryopreservation at low temperature slows or totally prevents unwanted physical and chemical change. The major disadvantage to using low temperature cryostorage is that it can lead to the crystallization of water, and thereby, this approach can create new and unwanted physical and chemical events that may injure the cells that are being preserved. Although the results achieved by slow freezing in many cases seem quite successful [1, 2], ice crystal formation still renders traditional slow-freezing programs generally less consistent in their clinical outcomes. Another downside to the slow-freezing approach is the time to complete such freezing procedures for human embryos, which can range from 1.5 to 5 h. This is due to the fact that the slow rate of cooling attempts to maintain a very delicate balance between multiple factors that may result in cellular damage by ice crystallization and osmotic toxicity. Traditionally, slow-freeze embryo cryopreservation has been a positive contributor to cumulative patient pregnancy rates, but ultimately, the limitations of current slow-rate freezing methods in ART have become more evident in the shootout with vitrification-based cryostorage.

Vitrification is one of the more exciting developments in ART in recent years that attempts to avoid ice formation altogether during the cooling process by establishing a glassy or vitreous state rather than an ice crystalline state, wherein molecular translational motions are arrested without structural reorganization of the liquid in which the reproductive cells are suspended. To achieve this glass-like solidification of living cells for cryostorage, high cooling rates in combination with high concentrations of cryoprotectants are used. A primary strategy for vitrifying cells and tissue is to increase the speed of thermal conductivity, while decreasing the concentration of the vitrificants to reduce their potential toxicity. There are two main ways to achieve the vitrification of water inside cells efficiently: firstly, to increase the cooling rate by using special carriers that allow very small volume sizes containing the cells to be very rapidly cooled; and secondly, to find materials with rapid heat transfer. However, one has to take into account that every cell seems to require its own optimal cooling rate, for example, mature unfertilized oocytes are much more sensitive to chilling injury than any of the cell stages of the preimplantation embryo. The earliest attempts using vitrification as an ice-free cryopreservation method for embryos were first reported in 1985 [3]. In 1993, successful vitrification of mouse embryos was demonstrated [4]. Furthermore, bovine oocytes and cleavage stages were vitrified and warmed successfully a few years later [5]. In 1999 and 2000, successful pregnancies and deliveries after vitrification and warming of human oocytes were reported [6, 7]. Since that time, and because it seems to be that both

entities appear to be especially chill-sensitive cells in ART, oocytes and blastocysts seem to receive a potentially significant boost in survival rates by avoiding ice crystallization using vitrification [8]. In general, vitrification solutions are aqueous cryoprotectant solutions that do not freeze when cooled at high cooling rates to very low temperature. Interest in vitrification has clearly risen as evinced by the almost exponential growth of scientific publications about vitrification. Vitrification is very simple, requires no expensive programmable freezing equipment, and relies especially 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.

During vitrification, by using a cooling rate in the range of 2,500–30,000°c/min or greater, water is transformed directly from the liquid phase to a glassy vitrified state. 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 [9, 10]. Vitrification of the aqueous solution inside cells can be achieved by increasing the speed of temperature change and by increasing the concentration of the cryoprotectant used. However, a major potential drawback of vitrification is the use of high concentration of cryoprotectant, and an unintentional negative impact of these cryoprotectants in turn can be their toxicity, which may affect the embryo and subsequent development in utero. It is therefore essential to achieve a fine balance between the speed of cooling and the concentration of the vitrifying cryoprotectants. 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 [11–13] have shown that the use of relatively high concentration of cryoprotectants such as 15% (vol/vol) 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.

Vitrification in principle is a simple technology, that is, potentially faster to apply and relatively inexpensive; furthermore, it is becoming clinically established and is seemingly more reliable and consistent than conventional cryopreservation when carried out appropriately [14, 15].

Cryoprotectant agents are essential for the cryopreservation of cells. Basically, two groups of cryoprotectants exist: (1) permeating (*glycerol, ethylene glycol, DMSO*) and (2) nonpermeating (*saccharides, protein, polymers*) agents. The essential component of a vitrification solution is the permeating agent. These compounds are hydrophilic nonelectrolytes with a strong dehydrating effect. Furthermore, these CPAs are able to depress the "freezing point" of the solution. Regarding the high concentration of cryoprotectant used for vitrification, and in view of the known biological and physiochemical effects of cryoprotectants, it is suggested that the toxicity of these agents is a key limiting factor in cryobiology. Not only does this toxicity prevent the use of fully protective levels of these additives, but it may also be manifested in the form of cryoinjury above and beyond that seen occurring due to classical causes of cell damage (osmotic toxicity and ice formation) during cryopreservation. In spite of this, the permeating CPA should be chosen firstly by their permeating property, and secondly on the basis of their potential toxicity. Because the permeating CPA is responsible for the toxicity (*the key limiting factor in cryobiology*), different cryoprotectants have been tested for their relative toxicity, and the results indicate that ethylene glycol (EG; MW 62.02) is the least toxic followed by glycerol. Additionally, these highly permeating cryoprotectants are also more likely to diffuse out of the cells rapidly, and the cells regained their original volume more quickly upon warming, thus preventing osmotic injury. Therefore, the most common and accepted cryoprotectant for vitrification procedures is ethylene glycol (EG). Today, EG is more commonly used in an equimolar mixture with DMSO. Often, additives are added to the vitrification solution such as disaccharides. Disaccharides, for example, sucrose, do not penetrate the cell membrane, but they help to draw out more water from cells by osmosis, and therefore, lessen the exposure time of the cells to the toxic effects of the cryoprotectants. The nonpermeating sucrose also acts as an osmotic buffer to reduce the osmotic shock that might otherwise result from the dilution of the cryoprotectant after cryostorage. In addition, permeating agents are able to compound with intracellular water, and therefore, water is very slowly removed from the cell. Hence, the critical intracellular salt concentration is reached at a lower temperature. Removal of the cryoprotectant agent during warming can present a very real problem in terms of trying to reduce toxicity to the cells. Firstly, because of the toxicity of the vitrification solutions, quick dilution of them after warming is necessary, and secondly, during dilution, water permeates more rapidly in to the cell than the cryoprotective additive diffuses out. As a consequence of the excess water inflow, the cells are threatened by injury from osmotic swelling. In this situation, the nonpermeating sucrose acts as an osmotic buffer to reduce the osmotic shock. 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 [16–18].

Pronuclear Stage Embryo Vitrification

Conventional cryopreservation of pronuclear zygotes (2PN) is well established in countries such as Germany where freezing of later stage human embryos is by law or by ethical reasons not allowed. The time to complete the conventional protocol to cryopreserved zygotes is 98 min. In Germany, the clinical pregnancy outcomes arising from the frozen-thawed 2PN cycles are about 18%, with an implantation of around 10% per embryo transferred. The time to complete vitrification of zygotes requires approximately 12 min. Recently, successful vitrification of 2PN embryos with a high survival rate is approximately 90%, cleavage rates on day 2 is greater than 80%, and blastocyst formation of 31%, and pregnancies were reported [19–22] (Table 59.1). Zygote vitrification implemented as a clinical setting can provide a clinical pregnancy rate of close to 30%, with an implantation rate of 17% [22]. The pronuclear stage appears well able to withstand the vitrification and warming conditions, which is probably due to the significant membrane permeability changes that occur postfertilization; such changes to the oolemma may also make it more stable and able to cope with the vagaries of the cold shock and striking osmotic fluctuations that occur during the vitrification process.

Cleavage Stage Embryo Vitrification

Reports of human embryo vitrification have been more frequent. Liebermann and Tucker [16] using either the cryoloop or the hemi-straw system (HSS) showed postwarming survival rates (after 2 h of culture of day 3 embryos where more than half of their blastomeres were intact) from 84 to 90% which was dependent on the carrier system used. There was a reasonable further cleavage and compaction rate of 34%. This finding supports previous reports in which high survival rates of eight-cell human embryos using 40% EG were documented [23]. In comparison to traditional slow-rate cryopreservation, a survival rate of cleavage stage embryos of 76% was reported with vitrification [24]. Recently, successful pregnancies and deliveries after vitrification of day 3 human embryos using the OPS have been reported [25, 26]. Their results showed a negative correlation between stage of development and survival, eight-cell embryos showed a higher survival rate (79.2%; 62/78) than did embryos with fewer than six cells (21.1%; 11/53) after vitrification [25]. Despite the fact that Liebermann and Tucker [16] achieved a promising postwarming survival rate, overall only about 34% of the surviving embryos had the developmental potential to reach the compaction stage. Recently, publications on cleavage stage vitrification provided good outcome data. Loutradi et al. [27] were performing a meta-analysis and systematic review by comparing traditional and vitrification protocols for cleavage stage embryos and found a survival rate of 84.0% vs. 97.0%. In addition, clinical pregnancy rates between 35 and 48%, with implantation rates between 15 and 39%, have been reported [28–31]. So, clearly, vitrification appears to have a positive impact on overall embryo utilization. A study on the neonatal outcome of 907 vitrified-warmed cleavage stage embryos found no significant increase in the congenital birth defect rate when compared with pregnancies using fresh cleavage stage embryos [32].

Table 59.1 2008/2009 Experience (SG/GRS) with vitrification for zygote cryostorage using EG/DMSO/sucrose protocol and open carrier system

Zygote warming cycles	7
Number of zygotes stored	69
Number warmed	47
Number survived	43 (91.5%)
Cleavage	43 (100%)
Number of ETs (day 3/day 5)	7 (6/1)
Embryos transferred (average)	16 (2.3)
Blastocyst vitrified	3
2PN utilization rate	19/43 (44.2%)
Clinical pregnancies	5 (71.5%)
Implantation rate	6/16 (37.5%)

Blastocyst Stage

Both natural and hormone replacement cycles seem to provide comparable levels of receptivity in naturally cycling women, though they differ in level of convenience. Regardless of the day of cryopreservation of the embryo (whether day 5, 6, or 7), at thawing/warming, blastocysts should be treated as if they had been frozen on the fifth day of development. Vitrification of blastocysts has been undertaken utilizing an "open system" (Cryotop; Kitazato BioPharma Co., Ltd., Fuji-shi, Japan) and a "closed system" (HSV (High Security Vitrification Kit); CryoBio System, L'Aigle, France) after a two-step loading with cryoprotectant agents at 24°C. Briefly, blastocysts were placed in equilibration solution, which is the base medium (HEPES-buffered HTF with 20% synthetic serum substitute (SSS)) containing 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) DMSO. After 5–7 min, the blastocysts were 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 45–60 s and transferred onto the Cryotop or HSV using a micropipette. Immediately after the loading of not more than two blastocysts in a 1 μL drop on the Cryotop, the carrier was plunged into fresh clean liquid nitrogen (LN2). After loading the embryos, the Cryotop was capped under the LN2 to seal and protect the vitrified material prior to cryostorage. In contrast, after loading the HSV, the straw was heat-sealed and then plunged in LN2, and stored the same way as the Cryotop [12, 13].

Table 59.2 Retrospective data from 2,907 patients (average age 33.6±4.9) with blastocyst cryopreservation by vitrification from January 2004 till December 2009

Day of development	Day 5	Day 6	Day 7	Total
No. of blastocysts vitrified	4,323 (43%)	5,478 (54%)	318 (3%)	10,119

Table 59.3 Retrospective data from the blastocyst cryopreservation program (Fertility Centers of Illinois, Chicago) where vitrification (VIT) technology was applied from January 2004 till December 2009

Technique	VIT
Patient's age (years)	34.6±5.1
No. of warmed cycles	1,754
No. of transfers	1,737
No. of blastocysts warmed	3,465
No. of blastocysts survived (%)	3,346 (96.6)
No. of blastocysts transferred	3,313
Mean no. of blastocysts transferred	1.9
No. of implantations (%)	998 (30.1)
No. of positive pregnancy/warm (%)	860 (49.0)
No. of positive pregnancy/VET (%)	860 (49.5)
No. of clinical pregnancy/warm (%)	748 (42.6)
No. of clinical pregnancy/VET (%)	748 (43.1)
Ongoing pregnancies/VET (%)	631 (36.3)
No. of live births	576 (277 boys and 299 girls)

Values are numbers unless otherwise described
VET vitrified embryo transfer

To remove the cryoprotectants, blastocysts were warmed and diluted in a two-step process. With the Cryotop or HSV submerged in LN2, the protective cap (Cryotop) or inner straw (HSV) were removed, and then both carriers with the blastocysts were removed from the LN2 and placed directly into a prewarmed (~35–37°C) organ culture dish containing 1 mL of 1.0 M sucrose. Blastocysts were picked up directly from the Cryotop and placed in a fresh drop of 1.0 M sucrose at 24°C. After 5 min, blastocysts were transferred to 0.5 M sucrose solution. After an additional 5 min, blastocysts were washed in the base medium and returned to the culture medium (SAGE Blastocyst Medium, Trumbull, CT, USA) until transfer.

Between January 2004 and December 2009, the *Fertility Centers of Illinois* "IVF Laboratory River North" (Chicago) has vitrified 10,119 blastocysts *without artificial shrinkage* before the cryopreservation procedure (Table 59.2). After 1,737 frozen embryo transfers (FET) including day 5 and day 6 blastocysts with a mean age of the patients of 34.6±5.1 years, to date, we have seen a survival rate, implantation, and clinical pregnancy rate per transfer (cPR) of 96.6, 30.1, and 43.1%, respectively (Table 59.3). In addition, in 482 FET using aseptic vitrification, 927 blastocysts were transferred with a survival, implantation and cPR of 97.5, 33.3, and 47.5%, respectively (Table 59.4). After 6 years of vitrifying blastocysts, the perinatal outcome is as follow: from 464 deliveries with vitrified blastocysts, 576 babies (277 boys and 299 girls) were born (Table 59.3). No abnormalities were recorded.

Table 59.4 Retrospective data from the blastocyst cryopreservation program (*Fertility Centers of Illinois, Chicago*) where aseptic vitrification (aVIT) technology was applied from June 2007 till December 2009

Technique	aVIT
Patient's age (years)	34.2±5.1
No. of warmed cycles	485
No. of transfers	482
No. of blastocysts warmed	959
No. of blastocysts survived (%)	935 (97.5)
No. of blastocysts transferred	927
Mean no. of blastocysts transferred	1.9
No. of implantations (%)	309 (33.3)
No. of positive pregnancy/warm (%)	260 (53.6)
No. of positive pregnancy/VET (%)	260 (53.9)
No. of clinical pregnancy/warm (%)	229 (47.2)
No. of clinical pregnancy/VET (%)	229 (47.5)
Ongoing pregnancies/VET (%)	221 (45.9)
No. of live births	115 (61 boys and 54 girls)

Values are numbers unless otherwise described
VET vitrified embryo transfer

Table 59.5 A comparison of retrospective data from the blastocyst cryopreservation program (*Fertility Centers of Illinois, Chicago*) of vitrified day 5 and day 6 blastocysts from January 2004 till December 2009

Day of development	Day 5	Day 6
Patient's age (years)	34.5±5.3	34.7±4.8
No. of warmed cycles	848	906
No. of transfers	845	892
No. of blastocysts warmed	1,732	1,733
No. of blastocysts survived (%)	1,673 (96.6)	1,673 (96.5)
No. of blastocysts transferred	1,652	1,661
Mean no. of blastocysts transferred	2.0	1.8
No. of implantations (%)	575 (34.8)[a]	423 (25.5)[a]
No. of positive pregnancy/warm (%)	487 (57.4)[b]	373 (41.2)[b]
No. of positive pregnancy/VET (%)	487 (57.6)[b]	373 (41.8)[b]
No. of clinical pregnancy/warm (%)	421 (49.6)[b]	327 (36.1)[b]
No. of clinical pregnancy/VET (%)	421 (49.8)[b]	327 (36.7)[b]
Ongoing/delivered pregnancies (%)	353 (41.8)	278 (31.2)
No. of live births	342	234

Values are numbers unless otherwise described
VET vitrified embryo transfer.
[a]$P<0.05$
[b]$P<0.01$

When the vitrified-warmed blastocysts were divided into day 5 and day 6 groups, the following data were gathered (Table 59.5): In 845 FET transferring day 5 blastocysts, the survival, implantation, and cPR were 96.6, 34.8, and 49.8% compared to 96.5, 25.5, and 36.7% of day 6 blastocysts. As shown in Table 59.5, implantation and cPR occurring in the day 5 blastocysts group were significantly higher than in

Table 59.6 A comparison of retrospective data from the blastocyst cryopreservation program (*Fertility Centers of Illinois, Chicago*) of aseptic vitrified day 5 and day 6 blastocysts from January 2004 till December 2009

Day of development	Day 5	Day 6
Patient's age (years)	34.2±5.5	34.3±4.4
No. of warmed cycles	235	250
No. of transfers	235	247
No. of blastocysts warmed	472	487
No. of blastocysts survived (%)	461 (97.7)	474 (97.2)
No. of blastocysts transferred	457	470
Mean no. of blastocysts transferred	1.9	1.9
No. of implantations (%)	181 (39.6)[a]	128 (27.2)[a]
No. of positive pregnancy/warm (%)	147 (62.6)[a]	113 (45.2)[a]
No. of positive pregnancy/VET (%)	147 (62.6)[a]	113 (45.7)[a]
No. of clinical pregnancy/warm (%)	129 (54.9)[a]	100 (40.0)[a]
No. of clinical pregnancy/VET (%)	129 (54.9)[a]	100 (40.5)[a]
Ongoing/delivered pregnancies (%)	125 (53.2)	96 (38.9)
No. of live births	78	37

Values are numbers unless otherwise described
VET vitrified embryo transfer
[a]$P<0.01$

the day 6 blastocyst group (χ^2; $P<0.05$; $P<0.01$, respectively). If we compare day 5 vs. day 6 outcome using a closed system, the following data in terms of survival, implantation, and cPR were observed: 97.7%, 39.6%, and 54.9% vs. 97.2%, 27.2%, and 40.5%, respectively (Table 59.6). As shown in Table 59.6, implantation and cPR occurring in the day 5 blastocysts group were significantly higher than transferring day 6 blastocysts (χ^2; $P<0.01$ for any comparison, respectively). After 3 years of vitrifying blastocysts using a closed system, the perinatal outcome is as follow: 115 babies (61 boys and 54 girls) were born (Table 59.4). No abnormalities were recorded.

Our data have shown that freezing at the blastocyst stage provides excellent survival, implantation, and clinical pregnancy [12, 13]. To achieve this data, the following points should be considered: (a) without a successful blastocyst vitrification storage program, extended culture should never be attempted; (b) the blastocyst is composed of more cells, and therefore, better able to compensate for cryoinjury; (c) the cells are smaller, thus making cryoprotectant penetration faster; and (d) on average, fewer embryos per patient are cryostored, but each one when thawed has a greater potential for implantation, often with an opportunity for an ET with a single blastocyst.

Furthermore, a vitrification solution with a mixture of 7.5% EG/DMSO, followed by a 15% EG/DMSO with 0.5 M sucrose step, is safe for clinical use, giving rise to healthy babies without abnormalities. Vitrification of blastocysts using an open or closed system (Cryotop or HSV) is effective for achieving high implantation and pregnancy rates as seen in fresh embryo transfers.

Although the outcome in terms of implantation and clinical pregnancy is significantly different when comparing day 5 blastocyst to day 6 blastocysts, our data should encourage cryopreservation of day 6 blastocysts as well. Based on the data presented, it is clear that the vitrification of day 6 blastocysts is of clinical value since it can result in live births. This observation is confirmed by Shapiro et al. [33] and Levens et al. [34]; they found that blastocyst development rate impacts outcome in slow cryopreserved blastocyst transfer cycles.

In conclusion, vitrification of human blastocysts is a viable and feasible alternative to traditional slow-freezing methods. The key to this success lies in the more optimal timing of embryo cryopreservation, for example, individual blastocysts may be cryopreserved at their optimal stage of development and expansion. In addition, the repeatedly discussed topic of using open systems (direct contact between cells and LN2) and the possible danger of contamination by bacteria, fungus, or different strains of virus from LN2 can be avoided by moving forward to a closed system providing lower cooling rates, but without a negative impact on the outcome.

Contamination of LN2: Open Versus Closed Systems

There are many potential advantages of vitrification in that it is an easy, cheap, fast, and an apparently successful cryopreservation method; however, there is one issue that is still up for debate. It has been shown that fungi, bacteria, and viruses are able to survive in liquid nitrogen (LN2) [35–40]. Given the direct exposure of the human cells as they are directly plunged into LN2 during the vitrification process, this therefore raises the question as to whether the LN2 has to be sterilized, as it may be a possible source of contamination for those cells. To this point, there has been no fungal, viral, or bacterial contamination that has been described from about 400 publications related to vitrification since the first report in 1985. Bielanski and colleagues [37] demonstrated a viral transmission rate of 21% to human embryos stored in open-freezing containers under experimental conditions of extremely elevated viral presence, while in contrast, all embryos stored in sealed freezing containers were free from contamination. Based on this observation, they proposed that the sealing of freezing containers appears to prevent exposure to potential contaminants. Commercial systems to purify LN2 by filtration have been developed; however, this technology to date has received little practical application in IVF laboratories that have active cryopreservation programs. While it is not totally clear that contamination is a real risk in everyday use of LN2, nevertheless, it may be prudent to consider routine sterilization of LN2 when open carrier systems are used for vitrification, followed by a sealing of that system for cryostorage. Further, there are

currently at least three "closed" sealed vitrification systems that are commercially available, with FDA clearance, that represent successful alternatives to open systems for embryo vitrification [13].

Conclusions and Future Directions

Vitrification is a very promising cryopreservation method with many advantages and an ever increasing clinical track record. There exist many variables that can profoundly influence the effectiveness and the survival rates of vitrified cells as shown in Table 59.2. A standardized vitrification protocol applicable to all stages of the preimplantation embryo may not be realistic because of (a) different surface-to-volume ratios; (b) differing cooling rate requirements between oocytes, zygotes, cleavage stage embryos, and blastocysts; and (c) 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.

An ideal strategy for vitrifying cells and tissue is to increase the speed of thermal conduction and to decrease the concentration of cryoprotectant, thus reducing potential toxicity. There are two main ways to achieve the vitrification of water inside cells efficiently. Firstly to increase the cooling rate by using special carriers that allow very small volume sizes containing the cells to be vitrified, and secondly to find materials with rapid heat transfer. For the adoption of vitrification in ART, as with all new technologies, there has been initial resistance, but as clinical data have 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.

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Directional Freezing of Reproductive Cells, Tissues, and Organs

60

Amir Arav

Abstract

The multi thermal gradient technique is based on the principles of directional freezing. The directional freezing process is based on controlling the ice crystal propagation through the sample by regulating the velocity of the sample through a predetermined thermal gradient. The directional freezing method permits a very precise and uniform cooling rate, which has allowed for successful freezing in the absence of liquid cryoprotectant agent (CPA). Ice formation occurs at the edge of the sample (seeding is initiated), and as the sample is pushed through the thermal gradient, the ice crystals propagate in opposite direction to the sample movement. This has enabled the successful cryopreservation of what has previously been regarded as impossible such as wild animal's sperm, human cartilage tissue, and large organs (sheep and human ovaries, porcine liver, and rat hearts).

Keywords

Multi thermal gradient technique • Directional freezing • Sperm freezing • Whole ovary freezing • Freeze-drying of cells reproduction

The multi thermal gradient (MTG) technique is based on the principles of directional freezing. The directional freezing process is based on controlling the ice crystal propagation through the sample by regulating the velocity of the sample through a predetermined thermal gradient. The directional freezing method permits a very precise and uniform cooling rate [1], which has allowed for successful freezing in the absence of liquid CPAs. Ice formation occurs as the edge of the sample (seeding is initiated), and as the sample is pushed through the thermal gradient, the ice crystals propagate in opposite direction to the sample movement. The ice crystal size and morphology are dependent on this velocity in an inverse proportion; that is, as the ice interface velocity increases, the size of the ice crystals decreases, and vice versa. The result is that the control of the unfrozen fraction size (the area between the ice crystals where the cells and solutes solidify) is controlled by the thermodynamics of the process and not by CPAs such as DMSO, glycerol, and their likes.

This has enabled the successful cryopreservation of what has previously been regarded as impossible such as wild animal's sperm, human cartilage tissue, and large organs (sheep and human ovaries, porcine liver, and rat hearts).

MTG Description

The MTG technique aims to overcome problems related to freezing of large volume of cells, tissue, and organs. The technology is based on a series of heat conductive blocks (usually built of brass or aluminum) arranged in a line, with a straight track running through the blocks. Along the blocks, different temperatures (T1, T2, T3, T4 in Fig. 60.1) can be set, thereby imposing a temperature gradient (G1, G2, G3). The blocks are separated by a gap (D1), and the temperature of the block on one side of the gap (T1) is above the freezing

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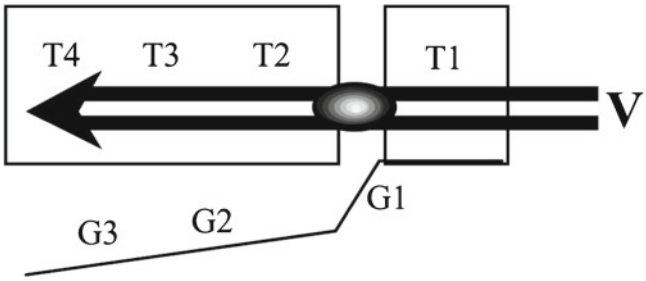

Fig. 60.1 Along the blocks, different temperatures (T1, T2, T3, T4) can be set, thereby imposing a temperature gradient (G1, G2, G3)

point temperature, whereas the temperature on the other side of the gap (T2) is below the freezing point temperature, thereby imposing a temperature gradient across the gap (G1). Biological samples to be frozen or thawed are placed inside the test tubes and are moved along the track at certain velocities (V). The samples are frozen at rates (cooling rate 1, 2, 3=G1, 2, 3×V) according to the specific protocols of the samples.

Sperm Freezing

Several freezing methods are in use for the cryopreservation of spermatozoa. Probably the most widely used one is the vapor freezing method during which the extended semen, packaged into 0.25- or 0.5-mL plastic straws, is held at a predetermined distance above liquid nitrogen for several minutes before being plunged into the liquid nitrogen for storage [2, 3]. Another method is known as the pellet method in which a small volume (usually around 200 μL) of extended semen is placed directly on carbon dioxide ice ("dry ice") and then stored in liquid nitrogen [4]. Other low-tech techniques include the dry-shipper freezing technique [5] or freezing in cold ethanol [6]. In more recent years, controlled rate freezing machines started penetrating the market [7]. In these machines, suitable for freezing extended semen packaged in straws, the rate of both the chilling and the freezing can be programmed and precisely controlled. The drawback of the various conventional freezing methods (vapor, pellet, controlled rate) is that ice crystal growth is uncontrolled in terms of both velocity and morphology, and the crystals may therefore disrupt and kill cells in the sample [8]. The alternative technique, which was described in a recent review as "the only recent significant advancement in semen preservation technology" [9], is the directional freezing technique. This technique uses the Multi Thermal Gradient device (MTG®; IMT Ltd, Ness Ziona, Israel) (Fig. 60.2). After the initial seeding stage, the semen sample is advanced at a constant velocity through a linear temperature gradient. The ice crystal propagation can thus be controlled to optimize crystal morphology and to achieve continual seeding and a homogenous cooling rate throughout the entire freezing process. Damage to the cells,

Fig. 60.2 Multi gradient thermal device in which large volume test tubes with samples are advanced at a constant velocity through a predefined temperature gradient to achieve optimal heat transfer and extracellular ice crystallization

even when freezing in large volumes of 8 mL, is thereby minimized [1, 10–12]. Several studies, conducted by us and others, that used this technology demonstrated the viability and fertilizing ability of frozen–thawed semen from a variety of species [11, 13–23]. The advantages of large volume freezing are apparent when one has to consider the storage space and costs of a large number of samples under liquid nitrogen over extended period of time. In studies conducted on bovine bull semen, we have demonstrated that semen frozen in this way can be thawed, packaged in insemination-dose straws, refrozen and later thawed and used for AI with acceptable fertility results, similar to those achieved in the conventional single-freezing method [11, 24]. Several studies on human sperm showed that some cells preserve their functional capacity even after several freeze–thaw cycles and can be used to fertilize oocytes through ICSI [25–27].

Recently, using the MTG, we have shown that it is possible to freeze cells in the absent of CPS. Freezing only with additives that are dry at room temperatures (such as sugars and proteins) allowing the freeze-drying of cells and storage at a dry state.

Freeze-Drying of Cells

For many years, scientists have been aspiring for the lyophilization of cells for the simplicity it allows in the storage and transportation of dried materials. In a recent publication, we have shown that freeze-dried granulosa cells that were stored for 3 years at room temperature preserved their DNA integrity and were capable to produce normal embryos after nuclear transfer [28, 29]. We have then continued on improving the viability and functionality of the dried cells after rehydration by adding an antioxidant to the lyophilization solution and by changing the freezing and drying parameters. This was done on MNC derived from human umbelical cord blood (UCB) units where we have achieved a viability rate of 85%

after rehydration with distilled water. Furthermore, when colony-forming unit assay was done, there was no difference between the numbers of colonies formed before freezing to those formed after freeze-drying and rehydration [30]. This assay has shown that the hematopoietic stem cells have maintained their capability to differentiate into different blood cells following freeze-drying.

Additionally, we have performed freeze-thawing and freeze-drying experiments with MNC derived from mice bone marrow which were transfused into sublethally irradiated mice. We have seen increased survival following injection of freeze-dried and rehydrated cells. The freeze-thawing experiments were done on blood from male mice that were injected into irradiated female mice. One month after the injection of the frozen–thawed cells, blood was taken from the female mice, and PCR was preformed showing the presence of Y chromosome. These preliminary findings suggest that the cells were capable to incorporate into the bone marrow and to form new white blood cells.

We have been able to lyophilize sheep leukocytes and show that their DNA is remained intact and can direct embryonic development after 3 years of storage at room temperature followed by rehydration with pure water. Human hematopoietic stem cells that were lyophilized and rehydrated with water were viable and have maintained their clonogenic capacity, showing that they were able to develop into all blood lineages. This was the first report to show cells that have undergone complete lyophilization and following rehydration have maintained not only their viability but also their functionality.

Whole Ovary Freezing

For many years, attempts to cryopreserve large organs have been ineffective due to problems associated with heat transfer [31] and the nonhomogeneous rate of cooling between the core and the periphery of the organ. To overcome these challenges, two methodologies have been proposed: the first is the MTG freezing technique [13, 17, 20] and the second is the vitrification methodology [32, 33]. Vitrification as a methodology for preserving large biological samples such as vascularized organs has many drawbacks: chemical toxicity and osmotic shock following exposure to very high (>50%) concentrations of cryoprotectant solutions, fractures caused to the vitrified organ by the vitrification procedure [34], and devitrification if the storage temperature is above the glass transition temperature [35].

The novel MTG methodology as shown by the experiments reported here and in other works [36] has solved this problem by maintaining a uniform cooling rate through the entire organ, by using two thermocouples, one in the ovarian cortex and the other in the center part of the medulla, that cooling rate is uniform throughout the periphery and the core of the ovary. Maintaining this uniform cooling rate was made possible by building our freezing devices based on two principles: (a) large mass of conductive material which enables rapid evacuation of latent heat being released due to crystallization in the freezing front, and (b) since the sample is moving through the temperature gradient at a controlled speed, we are able to create a very precise and uniform freezing front (the interface), which allows efficient removal of the latent heat.

The alternative to whole ovary is the cryopreservation of cortical strips. However, this method has major technical limitations since upon thawing and grafting of the cortical tissue, there is a vast loss of follicles during the period of ischemia and before the tissue becomes revascularized [37, 38], compromising ovarian function.

The successful demonstration of long survival—6 years—after whole ovary freezing and retransplant in a large animal does well for ongoing studies on whole human ovary cryopreservation and transplantation [39]. The information gathered from these animal experiments is very relevant to the human application since the sheep ovaries have strong similarities with young women ovaries displaying a high cortical primordial follicle density. There is hardly any data available on aged sheep ovaries since animals are normally culled long before 6 years of age [40]. However, it is the first time that long-term follow-up of whole ovary freezing and transplantation demonstrate a large number of follicles, as shown in the present study [41]. Cryopreservation of intact human ovary with its vascular pedicle is not associated with any signs of apoptosis or ultrastructural alterations in any cell types, confirming that whole-organ vascular transplantation may thus be a viable option in the future [42].

Summary

In summary, directional freezing is a promising technology which enables the freezing of large volume samples including large tissue and whole organs [36, 43, 44].

Concerning organ cryopreservation, with the advances in whole ovary, heart, and liver transplantation surgeries, the availability of an effective cryopreservation methodology is paramount.

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Ovarian Tissue Cryopreservation

61

Jennifer L. Kulp, J. Ryan Martin, and Pasquale Patrizio

Abstract

There is a large need for fertility preservation options for cancer survivors, yet at this time, ovarian tissue cryopreservation for fertility preservation remains experimental. Ovarian cortex banking may be offered to patients undergoing gonadotoxic therapy under an approved experimental protocol when ovarian stimulation and in vitro fertilization are not options. Cryopreservation and reimplantation of whole ovaries are areas where more research needs to be performed before it can be offered, even experimentally, to patients desiring fertility preservation. Future work will focus on clarifying patient selection for ovarian tissue cryopreservation. In addition, technical methods of cryopreservation of ovarian tissue should be optimized to enhance follicular survival. Lastly, surgical techniques for revascularization of thawed whole ovaries need to be perfected. The current outlook is hopeful that in the near future, ovarian tissue cryopreservation will be a viable treatment option for fertility preservation.

Keywords

Cryopreservation • Oocytes • Embryos • Ovarian tissue • Fertility preservation gonadotoxic therapy • Ovarian cortex banking

As cancer survival improves, women of reproductive age need options to preserve their fertility. Oncology treatments may involve gonadotoxic levels of chemotherapy or radiotherapy [1–5]. Freezing embryos is a traditional method of fertility preservation. This method 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. However, the disadvantage of this method is that a woman needs a husband, 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 cryopreserved oocytes are approaching that seen from cyropreserved embryos in some centers, and it is expected that soon this option will no longer be considered experimental [6].

This chapter will focus on the cryopreservation of ovarian tissue. While this method of fertility preservation is not as well established as embryo or oocyte freezing, it does offer some advantages. Contrary to embryo or oocyte cryopreservation, which requires synchrony with the follicular phase of the menstrual cycle and approximately 10 days of controlled ovarian hyperstimulation, resulting in high serum estradiol levels, ovarian tissue can be extracted and cryopreserved on short notice. It can be offered to women who need timely chemotherapy or who have an estrogen-responsive cancer such as some types of breast cancer. Ovarian tissue cryopreservation offers an advantage here as no pretreatment is

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needed, and a laparoscopy to harvest ovarian tissue can be performed at any point in a woman's menstrual cycle. It can also be offered to children, who are increasingly surviving from childhood malignancies, yet they may lose their fertility as a result of their chemotherapy [7–10]. The ability to preserve future fertility is important for quality of life in cancer survivors. Ovarian tissue cryopreservation may be the only viable option for children with malignancies who need to undergo gonadotoxic treatment.

Cryopreservation of Cortical Strips

The ovarian cortex contains a multitude of primordial and immature follicles. Cryopreservation of the ovarian cortex containing these large numbers of follicles is an emerging area of fertility preservation. The primordial and immature follicles are undifferentiated and are not metabolically active [11, 12]. In addition, the small water content, the high surface to volume ratio, and the absence of the zona pellucida may mean that these follicles are less susceptible to damage during the process of cryopreservation [3]. Ovarian cortical strips can be obtained via a same-day laparoscopy. The ovarian cortex can be harvested during laparoscopy in thin 1–3-mm strips, by removing approximately half the ovary in a block of cortical tissue or by taking small 5-mm biopsies [13, 14]. The use of cautery is avoided during ovarian cortex harvesting. Due to the unpredictable assessment of the risk for gonadotoxicity, some women can be expected to have some ovarian function remaining after chemo or radiotherapy; therefore, it is prudent to leave behind some of the ovarian cortex. With an experienced surgeon, the median operating time is 30 min. The complications from harvesting ovarian cortical strips via laparoscopy are minimal and not different from any other laparoscopic ovarian surgery [15]. However, there are challenges to freezing and reimplanting ovarian cortical strips. These thin strips are subject to ischemic damage both during cryopreservation and retransplantation. There may be poor permeability of the cryoprotectants and resultant freezing damage to the follicles contained within the ovarian cortex. Also, as these strips are frozen without their vascular supply, they rely on neovascularization for their posttransplant survival. Further depletion of follicles in fact occurs when the ovarian graft is reimplanted until a new vascular supply (generally after 5 days to a week) is established to perfuse the graft [16–19].

Ovarian cortical strips can be cryopreserved using both slow freezing and vitrification methods. The methods appear to be comparable in terms of preserving the follicles [20]. Success with cryopreserving ovarian cortical strips and autotransplantation was first demonstrated using animal models. In sheep, fertility was preserved after castration by autotransplantation of cryopreserved strips of ovarian cortex. After ovariectomy and transplantation of frozen–thawed ovarian cortex, FSH and LH levels returned to near normal for 60 weeks and estrous cycles resumed. Yet, a minority of primordial follicles, only 28% survived the process [17]. In the early 1990s, the birth of a lamb resulting from ovulation from a frozen–thawed ovarian cortical graft was reported [11]. Births of lambs were also reported after autotransplantation of cryopreserved hemi-ovaries, but again, only few follicles were seen on histologic examination of the frozen–thawed ovaries [21].

In humans, cryopreservation of ovarian cortical strips as a method of fertility preservation has emerged over the last 10 years. Ovarian cortical strips have been autotransplanted either into the pelvis (orthotopic) or outside the pelvis (heterotopic) [13, 22–27]. 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 but has also been done via laparotomy. The first live birth after orthotopic transplantation of cryopreserved ovarian tissue was reported in 2004 [24]. 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 which 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 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 more live births from the procedure are reported in the literature.

Since this first described successful orthotopic transplantation of frozen–thawed ovarian cortex, many others have been reported. The second successful case involved a 28-year-old with non-Hodgkin's lymphoma and ovarian failure after chemotherapy. She became pregnant after IVF and had a live birth when her cryopreserved ovarian cortex was thawed after 2 years and reimplantated on her ovary [25]. Overall, at least 12 live births have been described after orthotopic transplantation of frozen–thawed ovarian cortex [16, 24–31]. The patients were all in their twenties at the time of ovarian cortex freezing, and the cortex was frozen for a duration of 1–6 years prior to reimplantation. Some of these live births were after spontaneous conception and

others after in vitro fertilization. In 2010, Andersen et al. reported the first women to give birth to a second child after transplantation of frozen–thawed ovarian tissue. She conceived her first child after ovarian cortex reimplantation on her left ovary and delivered a healthy girl in 2007. She spontaneously conceived again that same year and went on to deliver a second healthy girl [32].

Heterotopic transplantation of ovarian cortex has also been described. In 2004, Oktay et al. described cryopreserving ovarian tissue from a 30-year-old woman with breast cancer prior to chemotherapy-induced menopause [23]. The cortex was then transplanted to the skin beneath her abdominal skin. After undergoing eight cycles of in vitro fertilization, one oocyte, out of 20 retrieved, fertilized normally and developed into a 4-cell embryo.

In 2006, a case was reported of a woman diagnosed with Hodgkin's lymphoma who had frozen–thawed ovarian tissue transplanted to a subperitoneal pocket on the lower abdominal wall. Twice an oocyte was retrieved from this location and fertilized by ICSI. After one of the embryo transfers, a biochemical pregnancy occurred [33]. Heterotopic transplantation of ovarian cortex tissue to other sites such as the forearm has also been described [13, 22, 23]. In monkeys, a successful heterotopic transplantation of fresh ovarian tissue, which has led to the birth of a healthy female after oocyte production, fertilization and transfer to a surrogate mother has been reported [34]. A live birth after heterotopic ovarian cortex transplantation has not yet been reported in humans, so this approach to ovarian tissue cryopreservation is nowadays rarely recommended.

Risks

One major concern of cryopreserving tissue from patients with a malignancy and then reimplanting this tissue is the risk of metastasis [35]. In one Danish study, in which females with leukemia had ovarian cortex cryopreservation, 6 of 8 patients had PCR evidence of the leukemic cells in the ovarian tissue [36]. Molecular markers of malignant cells allow detection of small numbers of cells by PCR which cannot be detected by histologic examination.

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 [35]. The viability of these malignant cells is unknown.

A study examined the presence of malignant cells in ovarian cortex of women with CML and ALL and found malignant cells present by PCR in 2 out of 6 patients with CML and 7 out of 10 patients with ALL. Further, when the ovarian cortex tissue of patients with ALL was xenografted into immunodeficient mice, the mice developed intraperitoneal leukemic masses [37]. These findings suggest that in patients with leukemia, reimplantation of ovarian cortex should not be recommended. Other malignancies seen in the reproductive years with a relatively high risk of ovarian metastasis are Burkitt's lymphoma and neuroblastoma. Reimplanting ovarian cortex may not be recommended in these malignancies [38, 39].

Advanced-stage breast cancer (stages III and IV) can metastasize to the ovary, and in patients with known metastatic breast cancer, it may be prudent not to cryopreserve and then reimplant cortical strips [14]. In contrast, more than 10 women with Hodgkin's lymphoma have received reimplanted ovarian cortex tissue, and no relapses have been documented so far [40–42]. All ovarian cortex tissue should be examined for malignant cells or minimal residual disease prior to being reimplanted [3, 43].

As ovarian tissue cryopreservation and reimplantation techniques are fairly new and not in widespread use as of this publication. The true long-term risk of metastasis in patients with malignancy who had ovarian cortex reimplanted remains to be seen.

Whole Ovary

Cryopreservation of ovarian cortical strips and then reimplantation lead to follicular loss secondary to ischemia. Transplantation of a whole ovary may result in less follicular loss as vascular supply to the tissue in theory can be more quickly established, decreasing follicular loss secondary to ischemia. The challenges of whole ovary cryopreservation include 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 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. Some authors reported on 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 [44].

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 resulted [45]. Recently, adult female sheep have become a standard model to study intact ovary cryopreservation [46, 47]. Arav et al. described

Fig. 61.1 Seeding of whole ovary in the glass tube prior to being loaded in the Multi-Thermal-Gradient device for cryopreservation

Table 61.1 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 [58], 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

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 [48]. 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 [49]. In 2006, a live-born lamb was reported after orthotopic microvascular reanastomosis of a whole cryopreserved ovary [50]. Whole ovary cryopreservation in the sheep has been attempted through both slow-cooling and vitrification methodologies [47, 51–53]. 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 [54].

In humans, the first report of cryopreservation and then thawing of a whole ovary was described by Martinez-Madrid et al. in 2004. 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 [55]. 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 [49]. 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. 61.1) [56]. 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 [57].

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 61.1 for a comparison of the cryopreservation of whole ovaries vs. cortical strips.

Conclusions

There is a large need for fertility preservation options for cancer survivors, yet at this time, ovarian tissue cryopreservation for fertility preservation remains experimental. Ovarian cortex banking may be offered to patients undergoing gonadotoxic therapy under an approved experimental protocol when ovarian stimulation and in vitro fertilization is not an option. Cryopreservation and reimplantation of whole ovaries are areas where more research needs to be performed before it can be offered, even experimentally, to patients desiring fertility preservation. Future work will focus on clarifying patient selection for ovarian tissue cryopreservation. In addition, technical methods of cryopreservation of ovarian tissue should be optimized to enhance follicular survival. Lastly, surgical techniques for revascularization of thawed whole ovaries need to be perfected. The current outlook is hopeful that in the near future, ovarian tissue cryopreservation will be a viable treatment option for fertility preservation.

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Part XI

Embryo Transfer

Assessment of Uterine Receptivity

62

Hakan Cakmak and Hugh S. Taylor

Abstract

Uterine receptivity plays a key role in the establishment of a successful pregnancy, and its impairment may limit the success of assisted reproductive technologies. Histological, morphological, and functional aspects of the endometrium were investigated in order to establish a test to assess endometrial receptivity and consequently to better predict pregnancy outcomes. Currently, histological dating and molecular markers of endometrial receptivity are poor predictors of pregnancy, and their assessment does not lead to improved pregnancy rates. Likewise, endometrial sonographic parameters used to evaluate uterine receptivity still lack specificity. However, endometrial biopsy and ultrasonography can be used efficiently to diagnose gynecologic disease that may affect uterine receptivity. Although hysteroscopy is the gold standard to evaluate endometrial lining and was shown to improve pregnancy outcomes after failed IVF cycles, it should not be offered as a first-line investigation in all subfertile women. In summary, there is no perfect diagnostic tool to assess endometrial receptivity. However, ultrasonography should be the first-line modality in detection of various endometrial pathologies, and efforts to optimize implantation should be directed to the treatment of possible underlying gynecologic condition disrupting endometrial receptivity. Conditions known to affect endometrial receptivity include hydrosalpinx, endometriosis, leiomyoma, adenomyosis, endometrial polyps, endometritis, and polycystic ovary syndrome.

Keywords

Implantation • Endometrial receptivity • Endometrial biopsy • Ultrasonography • Hysteroscopy

Embryo implantation represents the most critical step of the reproductive process and consists of a unique biological phenomenon. The blastocyst comes into intimate contact with the endometrium and forms the placenta that will provide an interface between the growing fetus and the maternal circulation. Successful implantation requires a receptive endometrium, a viable embryo at the blastocyst stage, and a synchronized dialogue between maternal and embryonic tissues [1].

The human endometrium undergoes a complex series of organized proliferative and secretory changes in each menstrual cycle and exhibits only a short period of receptivity, known as the "window of implantation" [2]. In humans, during a natural cycle, the embryo enters the uterine cavity 4 days after ovulation. The endometrium becomes receptive to blastocyst implantation approximately 6 days after ovulation and remains receptive for 4 days (cycle days 20–24) [3]. When implantation does not occur, a timely destruction of

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the fully developed endometrium leads to menstruation. However, if implantation occurs, the endometrium continues to grow and undergoes further morphological and molecular changes to support the growing embryo [2].

Implantation has three stages: apposition, adhesion, and penetration. Apposition is the unstable adhesion of the blastocyst to the endometrial surface. During this stage, the trophoblast becomes closely apposed to the luminal epithelium [4]. This is followed by the adhesion stage in which the association of the trophoblast and the luminal epithelium is strong enough to resist dislocation of the blastocyst by flushing the uterine lumen. This stage occurs on day 20–21 in humans and coincides with a localized increase in the vascular permeability at the site of blastocyst attachment. Local paracrine signaling between the embryo and endometrium triggers a stronger attachment [5]. Following adhesion, the embryo invades through the luminal epithelium into the stroma to establish a vascular connection with the mother, and this process is mainly controlled by trophoblasts; however, the decidua also limits the extent of invasion [5]. In response to this invasion and progesterone, the endometrial stromal cells and extracellular matrix undergo decidualization that is essential for the viability of the pregnancy.

Uterine receptivity plays a key role in establishment of successful pregnancies, and its impairment may limit assisted reproductive technologies (ART) success and contribute to the subfertility in several benign gynecologic disorders including endometriosis, hydrosalpinx, leiomyoma, endometrial polyp, adenomyosis, endometritis, and polycystic ovarian syndrome [6]. ART tools are now available that enable the selection of high-quality embryos, and protocols continue to evolve with the aim of achieving higher pregnancy rates, fewer multiple births, as well as healthy babies from genetically affected progenitors. However, despite these advances, implantation rates are still relatively low and have not increased sufficiently in the last decade to allow widespread adoption of single embryo transfer [7].

From a clinical point of view, practitioners need to have objective measurements to determine the probability for a healthy pregnancy, evaluating aspects of both embryo and endometrium, in a given menstrual cycle. This chapter aims to summarize the current knowledge about the available assessment methods of uterine receptivity.

Histological Assessment of the Endometrium

Histological analysis of the endometrium during the implantation window is limited by methodological issues, and there is no strong data to support the link between the endometrial receptivity and morphology within the same cycle.

Prerequisites for an adequate histological evaluation are exact timing of the biopsy within the cycle, selection of representative tissue of the functional endometrial layers, and optimal fixation and processing of the specimen. Timing of the endometrial biopsy must be synchronized to the LH surge, preferentially measured in serum.

Endometrium is a morphologically complex organ, encompassing several functional compartments with cellular heterogeneity that undergoes profound cyclic variations. In vivo studies are performed almost exclusively in nonconceptual cycles, and the quantification of profound cyclic variations in a reproducible manner is difficult. Noyes and coworkers examined the histological features of endometrial biopsies taken during 8,000 spontaneous cycles in 300 women and created the criteria for endometrial dating that previously accepted as gold standard approach for evaluating endometrial responsiveness and detecting endometrial abnormalities [8]. However, a number of weaknesses in Noyes' approach have been identified. Dating is most accurate in the early and late luteal phase, but not in the implantation window as very few histological parameters allow differentiation within the time span of the receptive endometrium [9]. The most notable weakness is high intra- and interobserver variability. Intraobserver variability has been shown to be highest among infertile women during the implantation window [9, 10]. Furthermore, ovarian stimulation in artificial cycles may lead to differences in the timing of endometrial maturation compared with natural cycles [11]. Additionally, histological dating does not discriminate between women of fertile and infertile couples and is therefore not a valid tool in routine evaluation of infertility or implantation failure [12].

Endometrial biopsy has proven to be useful for the diagnosis of intrauterine inflammatory states. Chronic endometritis has been associated with infertility and implantation failure because of the possible action of microbial products on the endometrial receptivity [13]. Patients with chronic endometritis have significantly lower clinical pregnancy and implantation rates (11 and 8%) compared with patients with negative biopsies (58 and 31%) [14]. Endometritis has been described in approximately 15% of patients undergoing a hysteroscopic workup before IVF and intracytoplasmic sperm injection and in up to 42% of patients with repeated IVF failure [15]. Moreover, a significant increase in pregnancy rates in subsequent IVF cycle was reported on completion of antibiotic treatment [14, 15].

Ultrastructural Assessment of Endometrium

Ultrastructural changes in endometrial epithelial morphology, characterized by the appearance of membrane projections called pinopodes, have been observed during the implantation window [16]. Pinopodes are progesterone-dependent organelles, appearing as apical cellular protrusions that

become visible between days 20 and 21 of the natural menstrual cycle, as shown by scanning electron microscopy in sequential endometrial biopsies [17]. Moreover, HOXA10, a homeobox gene whose expression is necessary for endometrial receptivity to blastocyst implantation, has an essential role in pinopod development. Indeed, blocking HOXA10 expression dramatically decreases the number of pinopods [18]. Although the mechanisms of action of pinopodes are still unclear, blastocyst attachment has been shown to occur at the top of endometrial pinopods [19]. Hypothetically, the receptors required for blastocyst adhesion may be located on the pinopod surface.

The clinical use of pinopod expression as a marker endometrial receptivity was supported by a study in which two consecutive endometrial biopsies were obtained during IVF mock cycles on days 6 and 8 of progesterone administration. The day of maximal receptivity, as established by the day on which mature pinopodes were detected, was determined for each woman [20]. In three-fourths of the patients, a new ideal embryo transfer day was determined and used in a subsequent transfer cycle. Of these women, 67% delivered a healthy baby. For one-fourth of women with no indication for modification to their transfer protocol, take-home baby rate was only 25%. However, the reproducibility and reliability of pinopod expression for testing endometrial receptivity was challenged by other studies. Poor intrapatient consistency in pinopode scores of postovulatory day 7 endometrial samples at consecutive cycles was demonstrated among infertile women [21]. Pinopode expression was not limited to the window of implantation but persists through to the end of the luteal phase [21, 22]. Another intriguing observation was that infertile women exhibit equivalent number of pinopodes as fertile controls at the time of implantation window [21–23]. Overall, there are mixed reports about the use of pinopods as an implantation marker in human; therefore, it should not be used as a predictor of the most receptive day of the cycle in clinical practice.

Functional Assessment of Endometrium

Implantation involves a complex sequence of signaling events that are crucial to the establishment of pregnancy. A number of molecular mediators, which are regulated with highest levels in the mid-secretory phase under the influence of ovarian hormones, have been postulated to be involved in this early feto–maternal interaction. These mediators embrace a large variety of interrelated molecules including adhesion molecules, cytokines, growth factors, and homeobox genes. The use of some of these molecules (such as αvβ3 integrin, MUC1, LIF, and HOXA10) has been investigated in order to identify a marker to better assess endometrial receptivity [24–27].

The Endometrial Function Test® (EFT) involves immunohistochemically staining of the endometrium with markers for the mitotic regulators cyclin E (the rate-limiting activator of the mitotic G1 to S phase transition) and p27 (an inhibitor of cyclin E) [28]. However, the use of endometrial receptivity markers has not been widely adopted clinically. αvβ3 integrin testing and the EFT are the only commercially available methods, and their clinical use has not yet been shown to improve pregnancy outcomes. Moreover, immunohistochemistry is subject to the same restrictions of histological assessment such as adequate timing of the endometrial biopsy and sampling accuracy. Overall, there is no perfect clinical assay to detect implantation defect so far.

Since morphological and single-gene approaches have failed to identify a marker that could be used in a clinical assay, research is now shifting toward microarray or global gene analysis [29]. DNA microarrays enable analysis of the simultaneous expression of thousands of genes in a single sample. Gene expression is a better marker of the biological phases and is likely a more reliable predictor of endometrial receptivity than morphology. A number of microarray studies have been performed during the implantation window, and all have reported genes that are strongly up- or down-regulated when the endometrium is receptive [30–34]. The information obtained from microarray studies is currently being used to form databases against which future biopsies can hopefully be compared to assist in patient management. The use of genome-wide arrays should eventually enable the verification of a cassette of genes that reliably predict or reflect endometrial receptivity as opposed to a single marker. As well as array technologies being used to study gene expression, methods are also being developed to study proteomic changes occurring during implantation. Endometrial secretion aspiration is one such approach and enables the measurement of protein changes in the uterine lumen during treatment cycles [35].

Morphological Assessment of the Endometrium

Ultrasonography

Ideally, a technique to assess endometrium and thereby predict endometrial receptivity must be easily performable within the daily clinical routine and would preferably be noninvasive. These requirements are met by ultrasonographic evaluation of endometrium.

Endometrial thickness is defined as the minimal distance between the echogenic interfaces of myometrium and endometrium, measured in the plane through the central longitudinal axis of the uterine body. Endometrial thickness is a relatively easily obtained and objective measurement with

low intraobserver and interobserver variability [36]. In premenopausal women, the endometrium is thinnest at the end of menstruation and appears as a single thin, well-defined line. The endometrial lining then expands under the influence of estrogen in the follicular phase of the cycle to the typical trilaminar hypoechoic appearance that measures typically between 6 and 10 mm in thickness [37]. After ovulation, the endometrium thickens to a diameter of 7–14 mm and becomes homogeneously echogenic under the influence of progesterone.

Compared with the natural cycle, sonographic endometrial lining is slightly thicker in patients receiving gonadotropin injections for infertility, and the thickness of the endometrium correlates with serum estradiol concentration [38]. The presence of endometrial polyps or intracavitary fibroids can falsely elevate the apparent endometrial thickness. Diethylstilbestrol (DES) exposure and intrauterine adhesions (Asherman's syndrome) will decrease the endometrial thickness.

Pregnancy rates after IVF are influenced only marginally by the endometrial thickness [38, 39]. Endometrial thickness has poor specificity and positive predictive value of 21 and 45%, respectively. In other words, many women with a normal endometrial thickness will still not be pregnant after transfer of embryos. The data extracted from the donor oocyte programs suggest that a pregnancy cannot be achieved if the endometrium thickness is below a certain critical cutoff limit. Although there are studies revealing that the thickness of endometrium for a successful implantation can be as thin as 4 mm [40–42] for the majority of the cases, at least 6 mm thickness of endometrium should be set forth as the prerequisite for a successful implantation [43, 44]. However, no correlation was demonstrated between endometrial histology and endometrial thickness either in spontaneous ovulatory cycles or in IVF patients [45, 46]. Fortunately, the proportion of patients with an unusually thin endometrium is low. If a pregnancy does not occur in a woman found to have a thin endometrium, then she should be assessed after the cycle for intrauterine pathology including Asherman's syndrome.

There is no consensus regarding the effect of abnormally thick endometrium on pregnancy rates during IVF cycles. Poor pregnancy and implantation rates were reported when the endometrial thickness was >14 mm on the day of hCG administration, or >15 mm on the day of embryo transfer, and >16 mm on the day of oocyte retrieval [47–49]. In contrast, others have found no effect of an increased thickness on pregnancy rates [50].

The ultrasonographic texture of the endometrium may have a prognostic value for implantation. In gonadotropin-stimulated cycles, echogenicity is usually assessed on the day of hCG administration. The "optimal" pattern is characterized by a multilayered hypoechogenic triple line pattern, but a hyperechogenic, homogenous solid pattern has also been described. Significantly reduced clinical pregnancy rate was demonstrated in patients with a solid endometrial pattern (34%) compared to those with a trilaminar pattern (47%) [41]. The interpretation of these findings is probably that an early luteal appearance is a sign of early progesterone production rather than any inherent fault with endometrial development. Hypoechogenic triple line pattern has a low positive predictive value (32%) for clinical pregnancy after assisted reproduction treatment [39]. In contrast, a hyperechogenic solid pattern has a reasonably high negative predictive value for pregnancy (75–89%) [39]. However, homogenously solid pattern should not be used an indication to cancel embryo transfers since patients with this endometrial pattern still have acceptable pregnancy rates after embryo transfer.

Transvaginal ultrasonography, especially when performed during the late follicular phase, provides excellent imaging of the uterus and of endometrial abnormalities that may affect the endometrial receptivity including endometrial polyps, leiomyoma, and adenomyosis.

Uterine fibroids can be detected by transvaginal ultrasound with great sensitivity. Leiomyomas are present in approximately 5–10% of women with infertility and are the sole factor identified in 1–2.4% [51]. Intramural and submucosal leiomyomas can distort the uterine cavity or obstruct the tubal ostia or cervical canal and, thus, may affect fertility [52]. Recent studies demonstrated that leiomyomas may also adversely affect the overlying endometrium and globally impair endometrial receptivity [53, 54]. Moreover, in the setting of a distorted uterine cavity caused by leiomyomas, significantly lower IVF pregnancy rates were identified [52, 55]. When myomectomies have been performed on women with otherwise unexplained infertility, the subsequent pregnancy rates have been reported to be 40–60% after 1–2 years [56, 57].

When evaluating the endometrium, endometrial polyps are not uncommonly found in infertile women. Endometrial polyps are identified by hysteroscopy in 16–26% of women with otherwise unexplained infertility, and the rate is much higher (46%) in infertile women with endometriosis [58, 59]. Endometrial polyps can be suggested by irregularities within the endometrial echo on traditional transvaginal ultrasound. Polyps are best detected by either saline infusion sonohysterography (SIS) or hysteroscopy as these have approximately equal sensitivity and specificity. The mechanism by which polyps may adversely affect fertility is also poorly understood but may be related to mechanical interference with sperm transport or embryo implantation. The only randomized trial examining the effect of polypectomy on pregnancy rate after intrauterine insemination demonstrated a statistically significant improvement in pregnancy rate in women who underwent hysteroscopic polypectomy compared with those who did not undergo polypectomy (63% vs. 28%) [60]. Three nonrandomized studies also found an association between

polypectomy and improved spontaneous pregnancy rates [61–63]. The effect of endometrial polyps on IVF remains unclear. Further, endometrial polyps <2 cm in size do not appear to impact IVF outcome.

A pathologic finding that can be mistaken for leiomyoma on ultrasound is adenomyosis. It is characterized by the presence of heterotopic endometrial glands and stroma in the myometrium with adjacent smooth muscle hyperplasia. Common ultrasound findings of adenomyosis include an enlarged uterus, poor definition of the endomyometrial junction, and the presence of myometrial cysts. Compared with fibroids, adenomyosis is characterized by more variable echogenicity and by a poorly defined lesion border. Ultrasound lacks adequate sensitivity and specificity identify adenomyosis. Magnetic resonance imaging using a T2-weighted image has improved test characteristics, but even MRI lacks both sensitivity and specificity to make it an ideal test for adenomyosis. An association between adenomyosis and subfertility has not been fully established. The presenting symptoms include a soft and diffusely enlarged uterus with menorrhagia, dysmenorrhoea, and metrorrhagia. Infertility is a less frequent complaint, since uterine adenomyosis is usually diagnosed in the fourth and fifth decade of life. However, when adenomyosis is encountered in younger reproductive age women, it is likely to reduce endometrial receptivity.

Transvaginal ultrasound may also be used to diagnose hydrosalpinges that are described as distally blocked, dilated, fluid-filled fallopian tubes with a heterogeneous spectrum of pathology. Two meta-analyses have shown that women with hydrosalpinx have lower implantation, pregnancy and delivery rates, and a higher incidence of spontaneous abortion after IVF-embryo transfer compared with women with tubal infertility of other causes [64, 65]. Furthermore, a prospective randomized clinical trial and a Cochrane review have demonstrated improved pregnancy and delivery rates with laparoscopic salpingectomy for hydrosalpinges prior to IVF, and the greatest beneficial effect was seen in women in whom the hydrosalpinges were both bilateral and visible by ultrasound [66, 67]. These findings suggest that, besides occluding the fallopian tubes, hydrosalpinx may also affect infertility through other mechanisms such as the impairment of endometrial receptivity by decreasing the expression of specific factors including αvβ3 integrin, LIF, and HOXA10 [68–70]. Furthermore, if the hydrosalpinges are removed, the expression of these markers returns to normal physiological levels in these patients [68–71].

Saline Infusion Sonohysterography

SIS is a procedure in which warm saline is instilled into the uterine cavity to provide enhanced endometrial visualization during transvaginal ultrasound. This technique improves detection of potential anatomic causes of reduced fertility, such as submucosal myomas, endometrial polyps, and intrauterine adhesions. The sensitivity, specificity, positive predictive value, and negative predictive value of SIS have been reported to be 98, 94, 95, and 98%, respectively [72]. In addition, it helps avoid invasive diagnostic procedures as well as optimize the preoperative triage process for women requiring therapeutic intervention. It is typically scheduled early in the follicular phase of the menstrual cycle, after cessation of menstrual flow and before day 10, as the endometrium is thin at this point in the cycle. Later in the cycle, focal contour irregularities of the endometrium may be mistaken for small polyps or focal areas of endometrial hyperplasia. Sonohysterography usually depicts leiomyomas and accurately assesses their location, size, and degree of intramural extension. It has added advantage of better estimation of the percentage circumference projecting into the endometrial cavity. This is important because if removal is planned, a greater than 50% projection of the leiomyoma into the uterine cavity suggests that hysteroscopic removal, instead of open removal, can be performed [73]. Therefore, in experienced hands, SIS may be an easy, safe, and well-tolerated alternative to diagnostic hysteroscopy in the initial evaluation of the uterine cavity.

Hysteroscopy

Hysteroscopy is considered to be the gold standard in the diagnosis of intrauterine pathology, including endometrial polyp, submucous leiomyoma, and intrauterine adhesions. Hysteroscopy can be performed without general anesthesia in an ambulatory setting with low cost, minimal morbidity, and inconvenience to the patient [74]. Furthermore, it gives the opportunity to correct the majority of the pathologies at the same time. There is no doubt that hysteroscopy should be performed when there is suspicion of intrauterine pathology at transvaginal ultrasonography or SIS. However, even when no abnormality is detected with these tools, subtle intrauterine pathologies have been noted in 18–50% of patients undergoing IVF at hysteroscopy [75]. In patients with at least two failed IVF or ICSI attempts, simple diagnostic or operative hysteroscopy before a subsequent IVF or ICSI treatment has shown to improve reproductive outcome [76, 77]. The higher pregnancy rates after hysteroscopy even in the absence of intrauterine pathology are a somewhat unexpected but biologically plausible observation. It is possible that cervical dilatation and/or an immunological mechanism triggered by the hysteroscopic manipulation or by the effect of the distension medium on the endometrium might play a role [78].

Conclusions

Receptive endometrium is indispensable for a successful pregnancy in ART cycles. Unfortunately, endometrial biopsy is invasive, and current histological dating and markers of endometrial receptivity are poor predictors of pregnancy. Evaluation using endometrial biopsy has not been shown to improve pregnancy outcomes. The need to evaluate endometrial development encouraged the use of ultrasonography as an alternative noninvasive method of assessment for uterine receptivity. Ultrasonographic endometrial thickness measurement and endometrial pattern determination were utilized to assess uterine receptivity and consequently to better predict pregnancy outcome in ART cycles. However, the endometrial sonographic parameters used to evaluate uterine receptivity still lack specificity, and its prognostic value in determining pregnancy rate is low. In contrast, endometrial biopsy and ultrasonography can be used efficiently to diagnose gynecologic disease that may affect uterine receptivity. Although hysteroscopy is the gold standard to evaluate endometrial lining and has been shown to improve pregnancy outcomes after failed IVF cycles, it should not be offered as a first-line investigation in all subfertile women.

In summary, there is no perfect diagnostic tool to assess endometrial receptivity. However, ultrasonography should be the first-line modality in detection of various endometrial pathologies, and efforts to optimize implantation should be directed to the treatment of possible underlying gynecologic condition disrupting endometrial receptivity.

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Single Embryo Transfer

63

Thorir Hardarson and Matts Wikland

Abstract

At the end of the day, single embryo transfer (SET) is not a big technical innovation, but rather more of an ideology, another way of defining success, not only through pregnancy rates in the fresh cycle. 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. Given that there are both pros and cons with SET, it is, however, likely that it will become more and more utilized as both patients and society realize the risk even with twins.

Keywords

Single embryo transfer • Multiple pregnancies • Double embryo transfer • Pregnancy results after SET • SET and DET rates compared

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 an increased number of multiple births, being 30% of all ART live births in the United States in 2006 [2]. In the United States, the *ASRM practice committee* [3] recommended that between one and five embryos be replaced depending on the maternal age 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 recently Turkey have promoted a stricter line in the form of legislation/recommendations greatly 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 a large variations between how SET has been implemented ranging from 2.8% in USA to 69.4% in Sweden [4]. These variations are likely to persist unless there are major changes in the way ART is viewed, funded, and legislated.

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 [5]. Despite that the majority of children born after multiple pregnancies are healthy, there are significant problems linked to multiple births both obstetrical and neonatal [6]. 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.

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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 USA to be 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€ [7].

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 [8]. 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.

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.

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 [9–15]. 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%) [8].

Several studies show, however, that a comparable ongoing clinical pregnancy rates can be achieved by transferring two embryos, one at a time [12, 13, 16]. However, such an approach requires a very good cryopreservation programs. Improved techniques for cryopreservation have dramatically increased both the survival rates as well as 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 [17–20]. 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.

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 singletons shown in a large follow-up studies of all IVF children born in Sweden between 1982 and 1995 [21], 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 [12]. 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 [12]. Without waiting for the final result from the abovementioned 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 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 (Fig. 63.1). During the same period, the delivery rate per transfer has remained stable around 26%. The MBR has, during the same period, dropped from 25 to

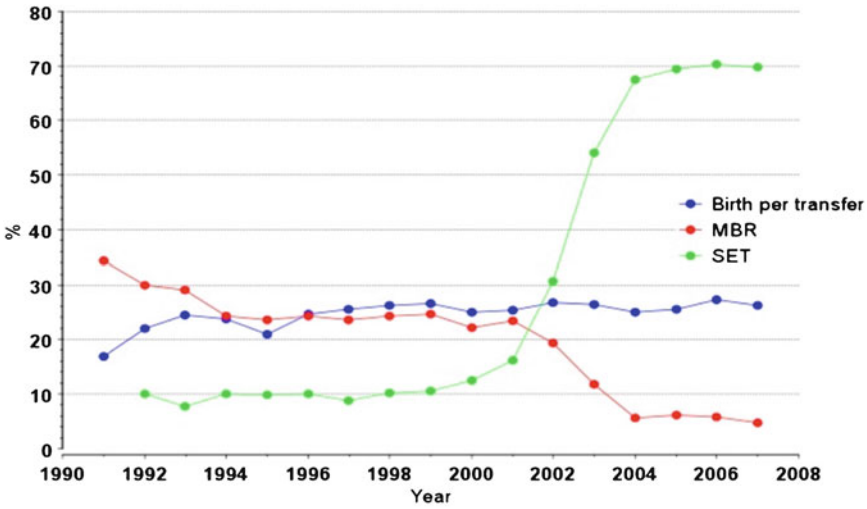

Fig. 63.1 Birth per transfer (standard IVF and ICSI), multiple birth rate (MBR), and single embryo transfer in Sweden in 1991–2007

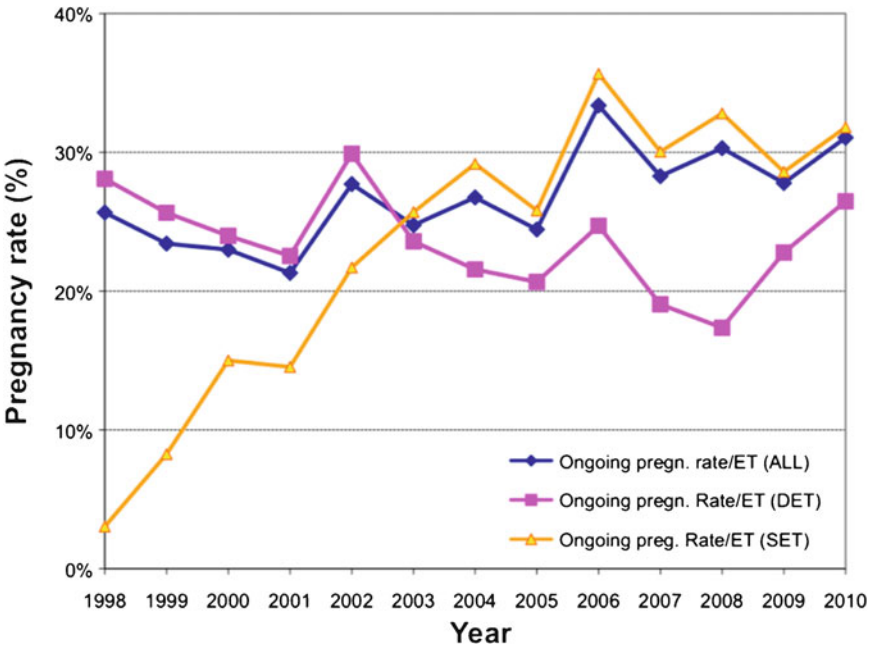

Fig. 63.2 Ongoing pregnancy rate after SET and DET at Fertilitetscentrum, Gothenburg, Sweden, during a 12-year period

5.7%. 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. 63.2). 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 [22]. Comparing the Swedish results with USA shows a continuous increase in delivery rate to 35%; however, the MBR is still around 30% (The Society for Assisted Reproductive Technology. National data summary. 2008 [30 October, 2010]; available from: www.sart.org).

The significant health consequences of such high MBR are obvious, and the cost for the American society is very high [2].

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 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 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.

Conclusion

At the end of the day, SET is not a big technical innovation, but rather more of an ideology, another way of defining success, not only through pregnancy rates in the fresh cycle. 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 [23]. Given that there are both pros and cons with SET, it is, however, likely that it will become more and more utilized as both patients and society realize the risk even with twins.

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Ultrasound-Guided Embryo Transfer (Abdominal/Vaginal): An Evidence-Based Evaluation

64

Ahmed M. Abou-Setta

Abstract

The literature generally supports the use of ultrasound guidance to assist clinicians during embryo transfer. However, the exact mechanism(s) whereby ultrasound-guided embryo transfer improves pregnancy rates remains unclear. Several theories have been proposed including (1) confirming the position of the tip of the embryo transfer catheter within the uterine cavity, (2) accurately determining the site of embryo deposition, (3) increasing the frequency of "easy" embryo transfers, and (4) avoiding endometrial indentation. Nevertheless, some clinicians might argue that the actual benefit of using ultrasound during embryo transfer is the ability to visualize the pelvic structures in real time. Whatever the underlying mechanism, or criticism of the procedure, the overall conclusion is that with most clinicians, using ultrasound will significantly improve their patients' pregnancy rates. Coupled with the fact that no study to date has shown any negative adverse events to the use of ultrasonographic guidance during embryo transfer, this technique has gained enormous popularity in the world of assisted reproduction.

Keywords

Ultrasound-guided embryo transfer • Assisted reproduction and ultrasound • Embryo transfer catheter • Transabdominal ultrasound during embryo transfer • Vaginal ultrasound-guided embryo transfer

Throughout time, fertility has been one of the most sought after aspects in societies. In addition to the natural wish to procreate, pregnancy and childbirth have been a highly sought after character in women of all walks of life. In ancient Egypt, women were graded according to potential fertility rather than their physical beauty, with the most attractive women being the fertile ones. In ancient Rome, fertility was such a key factor in the preservation of society that pregnancy and fertility were gracefully depicted on coins from the Roman Empire, a means to encourage fertility among couples.

Modern fertility practices can be traced back to the revolutionary work performed by Edwards and Steptoe in the 1970s. With the birth of the first baby in vitro, a new era was ushered in that was built on scientific discoveries and evidence-based decision making. Since their initial discoveries, major advancements have been made in ovarian stimulation protocols, embryo manipulation techniques, and in vitro embryo development. The result has been an increased probability of a live birth following embryo transfer from close to nil to over 25%. Even so, there has been a noticeable ceiling effect to success rates that does not seem to be related to the number or quality of embryos produced through in vitro fertilization since most women today will reach the embryo transfer stage with an adequate number of high-quality embryos. Therefore, the culprit preventing these healthy embryos from developing inside the maternal womb must lie later down the IVF chain. This weak link (embryo

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implantation) may be related to the technique used for embryo transfer and remains the rate-limiting step in the success of this form of therapy. It is estimated that up to 85% of the embryos replaced into the uterine cavity will fail to implant [1]. This makes the embryo transfer procedure a highly vital and, at the same time, highly inefficient step in IVF.

The pregnancy rate following embryo transfer is dependent upon multiple factors including embryo quality, endometrial receptivity, and the technique of the embryo transfer itself [2]. The aim of the embryo transfer procedure is to atraumatically and accurately place embryos within the uterus, in order to allow for proper implantation and fetal development. Years of experience and research have shown us that neither one of these goals are a given must during the transfer procedure. In addition, it is important to prevent iatrogenic complications of embryo transfer, such as vaginal bleeding and ectopic pregnancies. Ironically, just as Steptoe and Edwards were the first to describe a live birth following embryo transfer, they were also the first to describe an ectopic pregnancy following embryo transfer [3].

In recent years, the revelation of the importance of this stage of the IVF process has reflected in more research into ways of optimizing and standardizing the embryo transfer technique. Factors such as ease of the procedure [4], catheter choice [5], and dummy embryo transfer [6] have proven to improve the clinical outcomes, but nothing seems to have had such a major impact as the introduction of ultrasound guidance during embryo transfer.

The first reports of the beneficial effect of ultrasound guidance during the "blind" embryo transfer procedure were published over 20 years ago [7, 8]. Today, many centers and clinicians worldwide routinely perform all embryo transfer with the assistance of ultrasound imaging. Having said that, not all clinicians are believers, and there are a lot of skeptics over the true benefit of using ultrasound guidance during embryo transfer. This has been fueled by conflicting results from published clinical trials, with the majority concluding that ultrasound guidance improves the clinical pregnancy, and implantation rates, while some reports have shown no such improvement. In light of this controversy, it is important to critically view the relative efficacy of ultrasound guidance in an evidence-based fashion in order to appraise the best available evidence today for the use of ultrasonography during embryo transfer.

Abdominal Ultrasound-Guided Embryo Transfer

Two-dimensional (2D) transabdominal ultrasound is the most commonly used form of ultrasound guidance during embryo transfer. Even so, three-dimensional (3D) and four-dimensional (4D) ultrasound-guided embryo transfers have been reported [9]. Nonetheless, it is important to mention that the majority of the evidence in the medical literature relates to comparing the use of the 2D US-guided method.

All the preparatory steps (e.g., removing cervical mucus) before embryo transfer are performed just as with the clinical touch embryo transfer. Additionally, the woman is asked to fill the bladder to allow for proper visualization of the cervico-uterine axis. Once the clinician is ready to perform the actual transfer, a transabdominal ultrasound probe is placed on the lower part of the abdomen and adjusted by an ultrasonographer until a clear view of the reproductive path is evident. The clinician is therefore able to visualize the embryo transfer catheter as it traverses the cervical canal, internal os, and enters the uterine cavity. Once in the cavity, the embryos can be deposited as a specific distance from the uterine fundus. Moreover, the use of air bubbles to bracket the embryo containing media can give both the clinician and patient an accurate visualization of the final site of the embryos in the uterine cavity after the transfer catheter is removed.

Vaginal Ultrasound-Guided Embryo Transfer

Vaginal ultrasound-guided embryo transfer has been described in the literature but less frequently than with the use of abdominal ultrasonography [10, 11]. With the use of vaginal ultrasonography, the embryo transfer catheter is threaded through the external os into the cervical canal before the vaginal ultrasound probe is introduced. Further advancement of the catheter is then performed under ultrasound guidance.

The main advantage of this technique is that it allows the clinician to perform this procedure without the assistance of an ultrasonographer, as opposed to the abdominal ultrasound. Additionally, the patient does not have to fill the bladder, nor micturate immediately following the procedure. Advocates of this form of ultrasound-guided transfer claim that it is therefore more patient friendly by removing some of the discomfort associated with a full bladder.

Evidence-Based Evaluation on the Benefits and Drawbacks of Ultrasound-Guided Embryo Transfer

Systematic reviews and meta-analysis of randomized controlled trials have proven to be the highest level of evidence in the hierarchy of medical knowledge. The transparent nature and comprehensive coverage of a topic have made this form of research the most fundamental building block in search for the truth. There is an almost exponential growth in the number of published systematic reviews in the medical

literature, reflecting the growing recognition among clinicians and researchers of their importance for improving our understanding of health-related topics.

On the topic of ultrasonography during embryo transfer, there are several published systematic reviews of the evidence [12–16]. All the reviews were attempting to quantify the relative efficacy of ultrasound-guided embryo transfer compared with the standard "clinical touch" embryo transfer. Even so, it should be noted that the individual clinical queries were not precisely the same, neither were their search strategies nor inclusion criteria. Nevertheless, regardless of the differing methodologies, all the reviews came to the same final conclusion that there is adequate evidence to support the use of ultrasound guidance during embryo transfer.

The first systematic reviews using similar accepted methodologies identified eight prospective trials (four properly randomized and four quasi-randomized trials) comparing abdominal ultrasound-guided embryo transfer to clinical touch embryo transfer were published in late 2003 [12, 13]. The trials brought together data from 8,392 embryo transfer cycles (2,145 with ultrasound guidance and 2,051 without ultrasound guidance). The combined results showed that the clinical pregnancy rate was at least 17–32% better with ultrasound guidance than without. Additionally, the ongoing pregnancy rates were similarly higher with ultrasound than without (22%). Even so, the multiple pregnancy, miscarriage, and ectopic pregnancy rates were found to be similar in the two groups. On the bright side, the incidence of difficult embryo transfers was found to be lower with ultrasound guidance (9.1% compared with 15.1%).

It was several years later before the next systematic review on this topic was performed by our team in 2007 [14]. Again the methodology was built on well-defined and accepted practices in evidence-based medicine but differed slightly from the two previous reviews. We were able to include 20 randomized trials (5,968 embryo transfer cycles) in women undergoing ultrasound-guided (2,941 cycles) compared with clinical touch (3,027 cycles) embryo transfers. There was a general trend toward better results with ultrasound guidance, with only a limited number of studies demonstrating equivocal or better results with the clinical touch method. It may be assumed that studies with equivocal results demonstrate that some clinicians have excellent clinical sense and therefore nullifying any extra advantage provided by sonographic visualization. The pooled results from the properly randomized trials still demonstrated at least a 23% improvement in clinical pregnancy rates with the use of ultrasound guidance, with the number needed to treat (NNT) to obtain one additional clinical pregnancy using ultrasound guidance calculated to be 13 (95% CI = 10–19).

As with the previous reviews, the multiple pregnancy, miscarriage, and ectopic pregnancy rates were found to be similar in the two groups. Furthermore, the incidence of difficult embryo transfers and the need for instrumental assistance during transfer were confirmed to be lower with ultrasound guidance. Additionally, for the first time, the live birth rates were shown to be also improved with ultrasound usage. This is of importance since outcomes like the clinical pregnancy rates are only surrogate outcomes and not the final primary outcome in assisted reproduction.

One of the major points of concern in clinical trials and systematic reviews is the issue of sample sizes. It is theorized that smaller trials might not have a sufficient sample size to detect minor differences between the study groups (type II error) and will therefore present false equivocal results. The largest included trial ($n = 800$) in this review had enough power to detect an 8% difference in clinical pregnancy rates [17], while the overall review could detect an absolute difference of 3.3%.

In 2007, the first Cochrane systematic review [15] on the topic was published. This review included 13 truly randomized trials (2,714 women) comparing women randomized to ultrasound guidance (1,376 women) with clinical touch only (1,338 women) for catheter guidance during embryo transfer. This review was later updated in 2010 to reflect the most up-to-date information on the topic. The updated review [16] included 17 truly randomized trials (2,714 women) comparing women randomized to ultrasound guidance (1,254 women) with clinical touch only (1,218 women) for catheter guidance during embryo transfer.

Both the original and the updated reviews were performed according to the highest standards of evidence-based medicine and in accordance with the practice guidelines of the international Cochrane Collaboration. The results of the most recent update still demonstrate significantly higher incidences of pregnancy with ultrasound guidance, but no evidence of a significant difference in live birth rates was noted. Even so, the authors declare that the results from the trials were not homogenous and therefore should be interpreted with caution. Additionally, as with the previous reviews, the multiple pregnancy, miscarriage, and ectopic pregnancy rates were found to be similar in the two groups.

Clinical Discussion

It is evident from the aforementioned evidence that the literature generally supports the use of ultrasound guidance to assist clinicians during embryo transfer. However, the exact mechanism(s) whereby ultrasound-guided embryo transfer improves pregnancy rates remains unclear. Several theories have been proposed including (1) confirming the position of the tip of the embryo transfer catheter within the uterine cavity, (2) accurately determining the site of embryo deposition, (3) increasing the frequency of "easy" embryo transfers, and (4) avoiding endometrial indentation.

Nevertheless, some clinicians might argue that the actual benefit of using ultrasound during embryo transfer is the ability to visualize the pelvic structures in real time. Distorted pelvic anatomy, whether congenital or iatrogenic following surgery, can confuse the clinician into depositing the embryos at an improper site in the female reproductive tract. Furthermore, some clinicians have abandoned using measurements or information from dummy embryo transfers with the ultrasound guidance during embryo transfer. The reasoning is that the characteristics of pelvic structures tend to slightly change following ovarian stimulation, and therefore, any information passed on from the earlier stages of IVF may not be as accurate on the day of embryo transfer.

Others have been skeptical about the beneficial attributes of using ultrasonography during the transfer procedure. The argument is that experienced professionals have a strong clinical sense and therefore do not need to visualize the structures during embryo transfer. Additionally, the time to perform the transfer procedure is both increased and, in the case of abdominal ultrasonography, there is a need for an additional participant in order to perform the transfer procedure.

Whatever the underlying mechanism, or criticism of the procedure, the overall conclusion is that with most clinicians, using ultrasound will significantly improve their patients' pregnancy rates. Coupled with the fact that no study to date has shown any negative adverse events to the use of ultrasonographic guidance during embryo transfer, this technique has gained enormous popularity in the world of assisted reproduction.

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Cumulus-Aided Embryo Transfer

65

Firuza R. Parikh, Nandkishor J. Naik, Dattatray J. Naik, and Dhanajaya Kulkarni

Abstract

Although the factors that are involved in the regulation of blastocyst implantation are incompletely understood, increasing evidence suggests that growth factors and cytokines play a crucial role in the maternal–fetal interaction during embryonic implantation. It is known that the endometrium, preimplantation embryo, and the cumulus cells are a source of these cytokines and growth factors and their corresponding receptors at the developmental stage and also during the point of implantation. A better understanding of these factors during embryonic development and implantation could lead to improved in vitro culture conditions and enhance the outcome of human in vitro fertilization. In this chapter, we discuss the benefits of using cumulus cells in an embryo coculture.

Keywords

Cumulus • Coculture • Cytokines • Pregnancy rate • Growth factors • Cumulus-aided embryo transfer

The aim of all assisted reproductive technology (ART) procedures is to enhance implantation and therefore pregnancy rates. The limited implantation rate remains an unresolved problem in human reproductive medicine. Implantation failures can be attributed to embryo quality, inadequate uterine receptivity, or inadequate communication between the embryo and the endometrium. Evidence indicates that implantation is controlled by several local factors [1, 2].

Implantation of the human blastocyst is likely to be mediated via a number of signaling cytokine and adhesion molecules, although the precise molecular mechanisms involved in the human are incompletely understood and under investigation.

The procedure of intracytoplasmic sperm injection (ICSI) encompasses the enzymatic denuding of the oocytes off their cumulus cells, and hence, the potential positive effect of these cells on further development and quality of the embryo is lost. Data does indicate that embryos reach the blastocyst stage more frequently in a conventional in vitro fertilization (IVF) as compared to ICSI [3], implicating the lack of factors secreted by the cumulus cells in the latter.

Ovarian hormones induce marked morphological, physiological, and biochemical changes within the reproductive system. These changes in turn induce alterations in the biosynthetic activity and release of a myriad of locally produced proteins into the microenvironment of the reproductive tract. To harness the beneficial effects of the plethora of molecules, the use of cocultures rich in cytokines, growth factors, and angiogenic factors have been explored.

The coculture of human embryos during IVF has been reported to improve embryo quality, blastocyst development, implantation rates, and clinical pregnancy rates [4–6]. The process of coculture involves growing embryos in a culture medium on top of a proliferating monolayer of cells such as those from the fallopian tubes [7], endometrium [8], uterine fibroblasts [9], Vero cells [10], and cumulus cells [11].

The mechanism of action of the cocultured cells in enhancing the embryo quality and implantation ability

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includes detoxification of the media or the secretion of embryotropic substances such as cytokine and growth factors [12]. It has been postulated that the feeder cells metabolize the glucose present in the culture medium, thus allowing the embryos to be exposed to tolerable levels of glucose [13].

Several studies have demonstrated faster cleavage rates, less fragmentation, and better implantation rates using coculture systems [14]; they also increase blastulation rates to as high as 55–70% [15].

Previous studies have reported that this technique is useful in women with previous multiple failed IVF cycles and those with advanced age. In an attempt to improve the implantation rates, incessant search has been going on for a "glue" that will allow more intimate contact between the embryo and the uterine endometrium [16]. The use of autologous cumulus cells during embryo culture would probably fit the jigsaw. In a study, Parikh et al. have made use of the same cells, with an added innovative effort of cumulus-aided transfer (CAT); it involves cultivating the embryo on a layer of cumulus cells and performing embryo transfer along with some amount of the expanded cumulus cells, with enhanced implantation and pregnancy rates [17].

Although the factors that are involved in the regulation of blastocyst implantation are incompletely understood, increasing evidence suggests that growth factors and cytokines play a crucial role in the maternal–fetal interaction during embryonic implantation [18]. The preimplantation embryo produces several factors during its development to communicate and signal its presence to the maternal endometrium. Cytokines and growth factors and their corresponding receptors are the major candidates for these molecular events. The appropriate interaction between the preimplantation embryo and maternal endometrium is at least partially controlled by paracrine cytokines [19].

It is known that the endometrium, preimplantation embryo, and the cumulus cells are a source of these cytokines and growth factors and their corresponding receptors at the developmental stage and also during the point of implantation. A better understanding of these factors during embryonic development and implantation could lead to improved in vitro culture conditions and enhance the outcome of human IVF.

Procedure

Luteal phase suppression was followed by controlled ovarian stimulation with gonadotropins. The hCG trigger was given at follicular maturity, followed by transvaginal ultrasound-guided oocyte retrieval. ICSI was performed using standard protocols.

Cumulus Cell Culture

1. The cumulus cells are collected after hyaluronidase treatment.
2. The cumulus cells washed twice with Quinn's Advantage medium with HEPES.
3. The content from step 1 and step 2 are collected in falcon 2001.
4. Sample centrifuged at 1,300 rpm for 10 min.
5. The supernatant is discarded and mixed with the medium.
6. 5 mL of Quinn's Advantage medium with HEPES was added to the cell pellet.
7. The cells are mixed with the medium. Cell clumps are broken with the pipette in to single-cell suspension.
8. The centrifugation is carried out at 1,300 rpm for 10 min.
9. The supernatant is discarded.
10. The pellet is resuspended in Quinn's Advantage fertilization medium and cultured in a center-well dish falcon 3037 (1 mL of medium).
11. Next day, the medium is removed, and Quinn's Advantage cleavage medium was added. (Blood cells in the culture were removed by adding Quinn's Advantage medium with HEPES.)
12. On the day of coculture (day 01), fresh Quinn's Advantage cleavage medium is used. Equilibration was carried out for 2 h at 37°C temperature and 5% CO_2.
13. Every 48 h, the old medium was replaced with fresh medium till the time of embryo transfer.

Embryo Loading

Embryos were removed from the Petri dish containing the expanded cumulus cells and placed in a Petri dish containing Quinn's Advantage cleavage medium (which is used as embryo transfer medium) with 30 mg/mL of human serum albumin (Sage Bio Pharma). A cluster of approximately 30 μL of cumulus cells that was growing in close proximity to the embryo were collected with help of denuding pipettes and placed in the transfer medium. The embryos and the cumulus cells were loaded together into the embryo transfer catheter, and the contents of the catheter were released into the uterus over a period of 8–10 s.

Light Microscopy Evaluation of the Cumulus Cells

Cumulus cells were cultured from day 0 to day 10 and observed daily using an inverted microscope at ×4, ×10, and ×40 magnifications. Images grabbed using the image grabber software—Avanttec Image Plus™ Version 8.0.

Biological Background

The cumulus cells are known to secrete several factors like cytokines, growth factors, steroid hormone, and interleukins; the action of these varied factors can be harnessed for obtaining good-quality embryos and in turn enhanced pregnancy rates.

Cytokines

Cytokines are polypeptides of low molecular mass; their structures are often stabilized by N- and/or O-glycosylation and by intramolecular disulfide bridges. They play an important role in the communication between cells of multicellular organisms. As intercellular mediators acting in nanomolar to picomolar concentrations, they regulate survival, growth, differentiation, and effector functions of cells.

The mechanisms by which the maternal immune system does not reject the fetus during pregnancy despite the presence of paternal histocompatibility antigens are unclear. Th1 type cytokines, which includes interferon gamma (IFN-γ) and tumor necrosis factor beta (TNF-β), promote allograft rejection and compromise pregnancy; thus, the production of Th2 type of cytokines like IL-4, IL-10, and LIF (leukemia inhibitory factor), which inhibit Th1 responses, may allow allograft tolerance and fetus survival.

Some of the crucial cytokine molecules secreted by the cumulus cells in a coculture include LIF, vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), insulin-like growth factor (IGF-I), and interleukins such as IL4, IL-5, IL-6, and IL-10. The cumulus cells in a coculture also secrete the steroid hormone progesterone.

Leukemia Inhibitory Factor

LIF is a pleiotropic cytokine of the interleukin-6 family [20]. LIF is a secreted glycoprotein with a range of molecular weight forms, from 38 to 67 kDa, resulting from differential glycosylation of a protein of approximately 20 kDa [21]. LIF has been shown to be one of the essential cytokines for implantation [22]. LIF upregulates the trophoblast cells to produce chorionic gonadotrophin, which in turn is essential for the maintenance of the placental function. LIF gene mutations have been demonstrated to be the cause of implantation failure and consequently infertility in some women [23].

Vascular Endothelial Growth Factor

VEGF is a heparin binding, 45-kDa-homodimeric glycoprotein with key role as regulator of angiogenesis and vascular function in the human endometrium [24]. Angiogenesis is crucial for the development of endometrium as well as for embryo implantation and establishment of pregnancy. Abnormal expression of VEGF receptors during embryogenesis has been reported to result in a lethal phenotype [25].

TGF-β

The TGF-β super family is a large group of extracellular growth factors controlling many aspects of development and includes inhibins, activin, anti-Müllerian hormone, and bone morphogenic protein. At least 42 distinct mammalian dimeric proteins that share a similar structure are included in this family. TGF-β may play a role in human implantation via their stimulation of fibronectin or VEGF production [26] or by promotion of adhesion of trophoblast cells to the ECM [27].

TGF-β plays an important role in upregulating the crucial molecule LIF, inhibits the production of IL-2, and thus could be responsible for local immunosupression [28]. It increases the activity of matrix metalloproteinases MMP-9 and MMP-2 both of which are crucial proteins for the invasion and implantation.

Interleukins

Interleukins comprise a group of cytokines that participate in and also regulate pro- and anti-inflammatory immune responses. They possess a wide spectrum of biological activities like cell activation, cell differentiation, proliferation, and cell-to-cell interactions.

Interleukin-4 (IL-4)

IL-4 is a pleiotropic cytokine produced primarily by activated T lymphocytes, mast cells, basophils [29], and a subpopulation of activated T cells (Th2), which are the biologically most active helper cells for B cells. IL-4 is an anti-inflammatory cytokine that increase as pregnancy progresses [30].

Interleukin-5 (IL-5)

The biologically active form of IL-5 is an N-glycosylated antiparallel homodimer linked by disulfide bonds. Monomeric forms are biologically inactive. Variable molecular masses of the native protein are caused by heterogeneous glycosylation. Nonglycosylated IL-5 is also biologically active. These two interleukins are Th2 type of cytokines, and their important role is to stimulate the production of LIF.

Interleukin-6 (IL-6)

The IL-6 family consists of numerous cytokines, including LIF, IL-6, IL-11, neurotrophic factor, oncostatin M, and cardiotrophin 1 and plays important role in embryonic

implantation [31]. It is a multifunctional cytokine produced by many different cell types and regulates various aspects of the immune response, acute phase reaction, and hematopoiesis and has some functional redundancy with IL-11 and LIF. It has also been suggested that IL-6 may contribute to trophoblast growth and placental development in humans.

Interleukin-10 (IL-10)

Interleukin IL-10, also known as human cytokine synthesis inhibitory factor (CSIF), is an anti-inflammatory cytokine. This cytokine has pleiotropic effects in immunoregulation and inflammation. Along with TGF-β, it downregulates the expression of Th1 cytokines at the maternal fetal interface [32].

Progesterone

The steroid hormone progesterone is a key component in the complex regulation of normal female reproductive function. It is critical for the establishment and the maintenance of pregnancy, both by its endocrine and immunological effects. Progesterone plays a role in uterine homing of NK cells and upregulates HLA-G gene expression, the ligand for various NK inhibitory receptors. At high concentrations, progesterone is a potent inducer of Th2-type cytokines as well as of LIF and M-CSF production by T cells.

Progesterone plays a critical role in transforming the nonpregnant uterus into an enriched environment specifically suited for the developing embryo. Its role in inducing and maintaining the pregnant state is paramount and to a large extent determines the success of any given pregnancy in the steps following fertilization. It has been proposed that progesterone also plays an important role in the maternal shift of the Th1/Th2 balance [22].

Progesterone in the culture supernatants would promote the production of Th2 type of cytokines IL-4 and IL-5 and upregulates the production of LIF [22], and this is mediated through IL-4, as depicted below.

Progesterone induces IL-4, which further upregulates LIF, which modulates the trophoblast cells and aids in the embryo implantation. The window of implantation is established through the action of progesterone on estrogen-primed endometrium [33].

Clinical Outcome

Parikh et al. [17] have demonstrated an increase in pregnancy and implantation rates and higher-order multiple gestation rates using the cumulus-aided embryo transfer technique. Briefly, the results of the study are given in the Tables 65.1 and 65.2. In the study group, cumulus coculture and CAT technique was employed, and in the control group, only cumulus coculture was carried out.

Cumulus in Culture

The growth of the cumulus cells in culture is very rapid. Figure 65.1 shows increased colonization with cumulus cells in close proximity of the embryo. Cumulus cells after 24-h culture in Quinn's Advantage fertilization medium expand

Table 65.1 Results for demography and procedure variables

Parameters	Study group A	Control group B	*p* value
Average age	32.7 (±4.86)	33.0 (±4.44)	NS
Number of cycles	610	280	NS
Number of oocytes	4,697 (7.7 oocytes/cycle)	2,240 (8 oocytes/cycle)	NS
MII stage	4,135 (88%)	1,792 (80%)	NS
Fertilization rate (%)	72% (2,977/4,135)	71% (1,272/1,792)	NS
Cleavage rate (%)	92% (2,739/2,977)	90% (1,145/1,272)	NS
Average no. transferred	2.8	2.6	NS

Table 65.2 Results for pregnancy outcome

Parameters	Study group A $n=610$	Control group B $n=280$	*p* value
Pregnancy rate (%)	42% (256/610)	32% (90/280)	$p<0.01$
Implantation rate (%)	24% (406/1,689)	14.5% (105/725)	$p<0.001$
Multiple gestation (%)	39% (100/256)	31% (28/90)	NS
Higher-order multiple gestation (%)	18% (46/256)	4.4% (4/90)	$p<0.01$

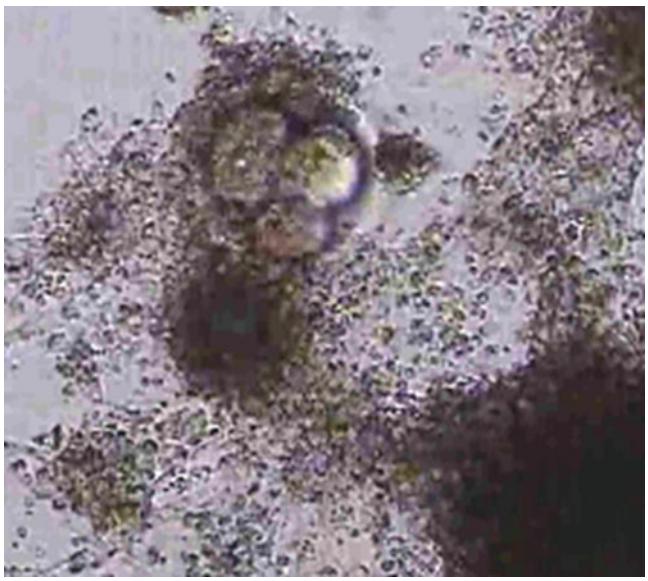

Fig. 65.1 Cumulus cells in close proximity of the embryo

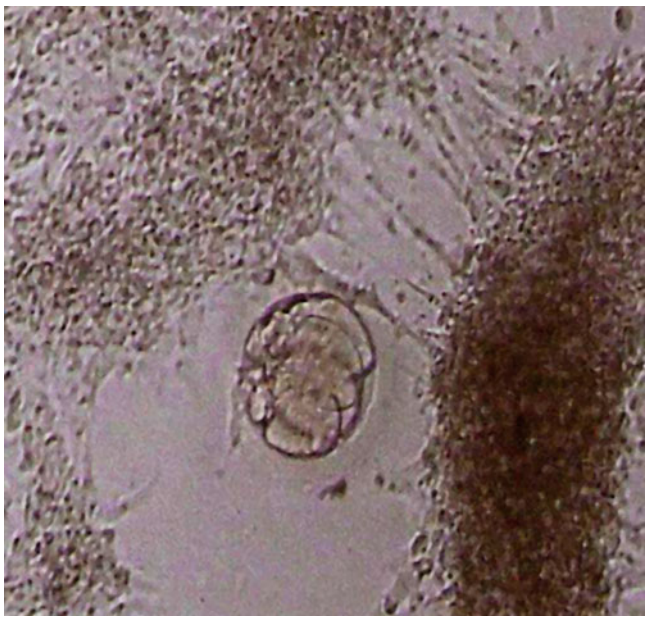

Fig. 65.3 Dendritic process of the cumulus

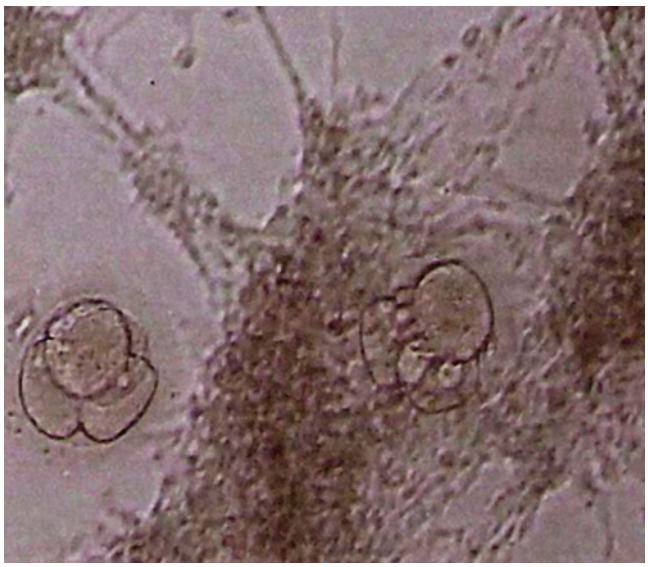

Fig. 65.2 Embryo immobilized by expanding cumulus

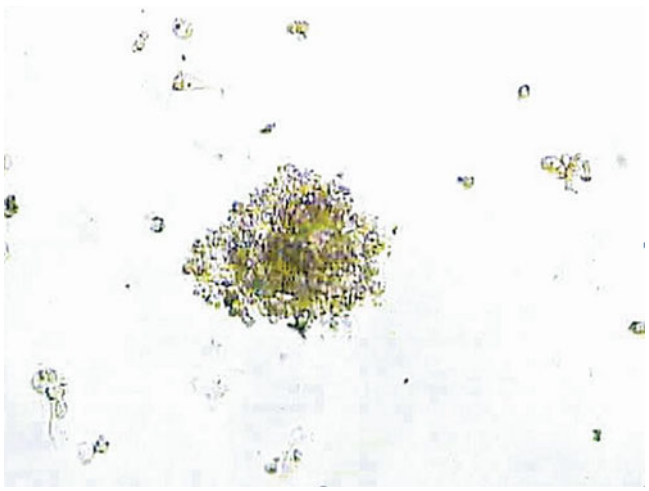

Fig. 65.4 Apoptosis of cumulus cells

into fibroblast-like cells and a vast expanse of dendrite-like processes. Between 36 and 72 h of oocyte retrieval, the entire Petri dish is covered with colonies of cumulus cells. They expand both below and above the embryo, engulfing it from all sides, as shown in Fig. 65.2. Figure 65.3 shows the dendritic processes of the cumulus cells extending towards the cleaving embryo, and apoptosis of the cumulus cells on day 10 of culture is shown in Fig. 65.4.

Summary

Successful implantation requires a receptive endometrium, a normal and functional embryo at the blastocyst stage, and a synchronized dialogue between maternal and embryonic tissues. Disturbances in the normal expression and action of the cytokines result in an absolute or partial failure of implantation and abnormal placental formation in human. As the development of culture media for ART evolves, paralleling other advancements in the field, we find ourselves at a critical

point at which increasing specialization and phase-specific design is the clear goal to help improve outcomes in human IVF. This leads us to the question, is there an ideal culture medium? Clearly, the goal is to emulate the in vivo environment as closely as possible. Because this milieu is dynamic compared with the virtually static in vitro environment, a single ideal ART medium seems elusive.

The benefits of using cumulus cells in an embryo coculture include the following:

- The cytokines synthesized by the cumulus cells play an important role in the development of the embryo in vitro and aids in the implantation.
- Production of progesterone by cumulus cells, an important steroid hormone of pregnancy, helps prime the endometrium and also creates a microenvironment conducive for implantation.
- The production of glutathione by cumulus cells, a very important antioxidant, neutralizes the reactive oxygen species (ROS); the culture media used in IVF is also a known source of ROS.

From a practical view, autologous cumulus cell coculture is easy to perform, less time consuming, has negligible risk for infection, and is a feasible alternative to optimize human embryo culture system. This technique of coculture is simple, avoids the use of heterogeneous cells, stimulates in vivo conditions, and enhances the quality of the embryo, making it competent to implant successfully.

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Part XII

Management and Regulation in the ART Laboratory

Data Management in the ART Laboratory: Requirements and Solutions

66

Timothy Brown and Bruce R. Gilbert

Abstract

The assisted reproductive laboratory has unique requirements for data management. There is a constant stream of data from both the andrology and embryology laboratories as well as those from reference laboratories. There is also quality control (QC) and quality assurance (QA) tests that need to be done at specified times with the results constantly being monitored by the laboratory staff. In addition, much of these data points need to be both entered and accessed in real time in a secure and verifiable way. Then, of course, there is the need for reporting on this data for clinical decisions, patient discussions, and to satisfy regulatory requirements. These requirements for our database solution create the need for a complex management solution. The challenge for database developers is to program all these features in the background and create a user interface that is simple and intuitive. This chapter discusses the basic components of a database design and the special concerns for the assisted reproductive technologies (ART) laboratory. We also introduce key terminology for discussions with developers. Our intent is to present information that will allow the reader to understand the essentials of data management, specific concerns for the ART laboratory, and current regulatory issues in order to better evaluate their current or future database solution.

Keywords

Database management solutions • Assisted reproductive technologies laboratory data • Tissue banking database • *Chain of custody* tracking of tissue • Data confidentiality in the ART laboratory

The assisted reproductive laboratory has unique requirements for data management. There is a constant stream of data from both the andrology and embryology laboratories as well as those from reference laboratories. There is also quality control (QC) and quality assurance (QA) tests that need to be done at specified times with the results constantly being monitored by the laboratory staff. In addition, much of these data points need to be both entered and accessed in real time in a secure and verifiable way. Then, of course, is the need for reporting on this data for clinical decisions, patient discussions, and to satisfy regulatory requirements. These requirements for our database solution create the need for a complex management solution. The challenge for database developers is to program all these features in the background and create a user interface that is simple and intuitive.

This chapter discusses the basic components of a database design and the special concerns for the assisted reproductive technologies (ART) laboratory. We also introduce key

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terminology for discussions with developers. Our intent is to present information that will allow the reader to understand the essentials of data management, specific concerns for the ART laboratory, and current regulatory issues in order to better evaluate their current or future database solution.

Databases

A database is a software tool to store data in such a manner that the data can be entered and recalled easily, securely, and reliably. Initially, word processors or spreadsheets were used to store information. However, users quickly outgrew this because such information is difficult to search and update as the quantity of data multiplies exponentially. A database provides the framework to store such information as data elements, so the user can easily access it. However, it is up to the user to design the framework of the database so that it allows ease of data entry and ease of data recall.

For example, let us say you receive a monthly summary of all your patients' lab results as values in a spreadsheet. How would you find the history of a single patient's lab values for the past 2 years? To do this using your spreadsheet, you would need to open up the 24 spreadsheets you have received and find the results of this single patient and then copy the values and place it into another spreadsheet to show the history. That would be, to say the least, time-consuming. However, if the database framework was designed to include the patient name, date of lab test, and name of the lab test, the user would only need to query the database (fancy word for find) for these values for a specific patient name, and the data would instantly be returned.

A database is therefore an integrated collection of logically related records or files consolidated into a common pool that provides data for one or more multiple uses. The database stores, manages, and retrieves data via the use of tables. If you are familiar with spreadsheets, then you may unknowingly be familiar with what a table is and therefore how databases are structured. A worksheet represents a table, and each worksheet can have many columns (fields) and rows (records). Like a spreadsheet, a database can have many worksheets (tables).

The way data is stored in a database is really important in order to retrieve and report on such data. In general terms, every time we see a patient, we do not create a new patient chart, we just add new information to the chart. The same logical progression occurs with a database. It gets better. A well-designed database will allow us not only to view the patient records but also to cross-reference this patient with other like patients. We also do not have to fill out repeating information each time we enter new data on a patient. For example, patient demographic information only needs to be entered once for a patient since it has been previously entered into the database.

There is, however, an important caveat. The value of the information recalled from a database is only as good as the information entered into the database. A well-designed and normalized database can prevent input of erroneous data by incorporating what is termed business logic. Business logic, built into the database through a process known as programming, provides a database with intelligence. It requires the database to act in a defined way. For example, when we are entering a new patient to the database, we might want to require the following fields to be completed: first name, last name, address, date of birth, and medical record number. If anyone of these fields is not completed on data entry, then we can write a short program, often termed a script, to report this error to the user. The programming, or built-in logic, can be quite different depending on the patient population. Take for instance a field containing the date of birth. If all our patients are adults, we might require that this field must be between 18 and 120 years. However, for a pediatric patient population, the logic might be between 0 and 18 years. In the end, what the business logic does is based on your particular needs.

There are less than a hundred companies that design database platforms: mySQL, Oracle, DB2, Microsoft SQL, PostgreSQL, Access, FileMaker, and 4D are a few of the large platforms offered. There are thousands of companies that create solutions for customers based on these database platforms. Almost all of these databases use Structured Query Language (SQL) to store and retrieve data. To be able to view this data, you need a user interface. There are two principle types of user interfaces, graphical user interface (GUI) and Web-based user interface (WUI). There are numerous programs that developers utilize to create a user interface.

Interoperability

ART laboratories utilize a multitude of software systems. These might include practice management systems (PMS), electronic health records (EHR), and laboratory information systems (LIS), as well as multiple data streams entering their facility from outside laboratories and regulatory agencies. The ability for these systems to talk to each other is extremely important. Doing so saves staff time by reducing the need to enter the same data in multiple systems and allowing for real-time access to data, as well as providing for a single interface for all data. Interoperability [1] is "the ability of two or more systems or components to exchange information and to use the information that has been exchanged." In order to achieve interoperability between systems, there must be a standard language the systems can understand. The standard used in health care is called "HL7." There are two version of this standard being employed by institutions, version 2.x and

version 3.x. The majority of messaging being employed today uses version 2.x. There has been a slow adoption rate of the newer version 3.x standard created in 1995.

Many systems on the market are HL7 compliant. In other words, they can accept and transmit HL7 messages between other HL7 compliant systems. It is important to know what version they support to know the interoperability with other systems. Although HL7 transactions are a standard, implementation is certainly not turnkey. The HL7 standard provides 80% of the framework for implementation, leaving the other 20% for a customized interface. This customized portion is where a software developer can spend a considerable amount of time building the interface.

System integration is often confused with system interface. There is a significant difference between the two and is relevant to HL7 transactions. If two systems are *integrated*, that generally means the data is only stored in one system and is accessed by the other system. With system integration, there is no confusion as to what is the current data. When systems are *interfaced*, the data is replicated from one system to the other, and therefore, there is more than one source for that data. Having the same data in multiple places, as occurs when systems are interfaced, can cause data to be out of sync, causing confusion on what is the most current data.

ART Laboratory-Specific Concerns

Reproductive Tissue Storage

All ART laboratories need to manage the storage tanks that contain reproductive tissues (sperm, oocytes, and embryos). Good clinical practice (GCP), as well as many regulatory agencies, requires *chain of custody* tracking of the movement of vessels stored in these tanks. In software jargon, this is referred to as an audit trail. This can be done electronically or on paper. However, any system you implement needs to document multiple data points each time a vessel (i.e., vial or straw) is moved, used, destroyed, or released. There also needs to be rules for identifying the transaction and verifying the user's privileges for that transaction, meaning that only authorized personnel should be able to perform functions in the system that is within their scope of work. When receiving vessels from another lab, store the receiving labs demographic and tissue information. When receiving sperm vessels, the sperm analysis should be entered into the system if available. This allows easy comparison to the sperm processing once used.

Your standard operating procedure manual (SOP) will need to be reviewed and revised based on the business logic of the software you selected. This is often overlooked, but it is critical that the SOP and software solution are consistent. For example, if, prior to implementing a software system that manages the specimens in tanks, the rules are that a lab technician can prepare the vessels to be sent and the lab director must sign the release form prior to placement in the Release Binder. The new rule may be that the lab director must electronically sign the database record *prior* to the release of the vessel.

The tracking of media that touches any reproductive tissue is regulated. Each time a solution (e.g., media or reagent) comes in contact with the reproductive tissue, that solution needs to be identified and tracked along with the vessel. This entails tracking a number of data points, for example, media name, manufacturer, lot number, date of receipt, and date of expiration, to name a few. The ability to quickly find the vessels and corresponding patients associated with a given solution lot number is particularly important in the rare instance of a manufacturer recall.

IVF Cycle Tracking

There is also the need for the IVF cycle itself to be tracked. Medications and lab values for each stimulation day need to be stored within the database. Once oocytes are retrieved, patient retrieval containers need to be verified. In fact, verification occurs at many steps during the IVF cycle and needs to be tracked and achieved for both internal and external audits. This will involve the development of systematic way of organizing the data and providing for verification by a number of staff as specified in the facilities SOP. This organization of data and verification can also be extended to the processes of insemination, retrieval, and transfer, with each of these processes having unique needs. For example, timing the hCG injection and retrieval not only requires following embryo development by ultrasound and hormone levels but also needs to be coordinated with staff and procedure room availability. This can be efficiently managed with real-time processing. The system can also provide for local or remote access to the database by all staff through secure portals.

The use of a sophisticated software solution can also decrease staff time and decrease errors by reducing the multiple entry steps required during the IVF process. One significant way to do this is by entering live data. Avoiding duplication in data entry is paramount. With lab space limited, quick data entry into a tablet PC at the "bench," we have found to be critical to system adoption by the IVF staff. With live bench entry, daily embryo development and grading can be rapidly entered into the database and immediately available to all the IVF staff.

Reporting of IVF data is required by both federal and most state regulatory authorities. In laboratories using paper and pen entry or even spreadsheets, this often takes hundreds if not thousands of staff hours each year to complete. The amount of data required to be tracked and reported on makes

paper and pen or even a standard spreadsheet approach difficult if not impossible to use. It then becomes a manual process to extract the required data for reporting purposes. The time and cost savings of a database solution that has reports scripted to provide the required information on demand are obvious.

Regulatory Reports

The 1992 Fertility Clinic Success Rate and Certification Act [2] requires fertility clinics to file annually the Assisted Reproductive Technology Report (ART Report) with the Centers for Disease Control (CDC). There are two methods of submitting this data to the CDC. Clinics can submit directly to CDC, or if they belong to the Society of Assisted Reproductive Technology (SART) of the American Society of Reproductive Medicine (ASRM), they can enter data to the SART online system. SART then submits their data to the CDC. Many clinics, however, complete this report manually and file with the CDC.

These reports can be submitted through the Internet. However, these reports are complex and require extensive programming and testing to assure accurate transmission of data. Therefore, when reviewing clinical system for your practice, you should make sure that the system being considered can submit data to the agencies that regulate your ART laboratory. Automating those reports can result in substantial savings in staff and financial resources.

Data Access

Having unrestricted access to your own data may seem obvious. However, commercial databases are often "locked down," allowing access to only the areas the vendor allows. This restriction is particularly onerous when you want to make impromptu searches of your data, possibly for a question a patient asked you or for a presentation you need to make. The term "data mining" refers to the process of analyzing data to uncover patterns. Typically, databases are the primary software solution to uncover these patterns. However, many systems do not provide for full access to your data, making data mining difficult. Without this access, your ability to search and report is limited. In addition, the ability to create your own reports is often difficult or impossible without customization by the vendor. Although most database solutions provide for exportation of data to a spreadsheet format (.csv, .exl, etc.), it still requires time and effort on the part of the user and would need to be repeated each time a report is desired. What would be ideal is a dialogue-driven report generator. Most vendors provide consulting services to assist in developing customized dialogue-driven reports. However, the rates for these services are typically higher than that for programming since developing a report requires a working knowledge of the structure of the database as well as the need to test the report for errors. Therefore, it might be well worth the cost to have the vendor create reports that are required.

Regulatory Requirements

A number of federal and state regulatory requirements need to be considered when evaluating software systems. These include the Health Insurance and Portability Accountability Act of 1996 (HIPAA) [3, 4], HITECH Act of 2009 [5], 21 CFR Parts 1270 and 1271, plus state regulations by the Department of Health.

HIPAA Security Rule

The Security Rule is a key part of HIPAA, which was a federal legislation that was passed into law in August 1996 [4]. The Final Rule on Security Standards was issued on February 20, 2003 and took effect on April 21, 2003. Compliance dates were April 21, 2005 for most covered entities and April 21, 2006 for "small health plans" (defined as health plans having annual receipts of $5 million or less). The Security Rule complements the Privacy Rule. While the Privacy Rule pertains to all protected health information (PHI) including paper and electronic, the Security Rule deals specifically with electronic protected health information (ePHI). It lays out three types of security safeguards required for compliance: administrative, physical, and technical. *Administrative Safeguards* refer to policies and procedures designed to clearly show how the entity will comply with the act. *Physical Safeguards* involve controlling physical access to protect against inappropriate access to protected data. *Technical Safeguards* refer to controlling access to computer systems and enabling covered entities to protect communications containing PHI transmitted electronically over open networks from being intercepted by anyone other than the intended recipient.

The primary security safeguard that software systems need to satisfy are the technical safeguards. The HIPAA regulations were designed in such a way that compliance can vary from practice to practice, and because of new technology and standards, a system that is compliant today may not be tomorrow. So, what is a practice to do? First, there is no such thing as a compliant or certified system. The Health and Human Services is responsible for enforcing HIPAA regulations. There is no governing body or company entrusted to certify individuals as "HIPAA Certified" or companies or products getting "official HIPAA certification." Therefore, it is up to you to do your homework. Tables 66.1 and 66.2

Table 66.1 Required technical safeguards

1. Access control: implement technical policies and procedures for electronic information systems that maintain electronic protected health information to allow access only to those persons or software programs that have been granted access rights as specified
2. Unique user identification: assign a unique name and/or number for identifying and tracking user identity
3. Emergency access procedure: establish (and implement as needed) procedures for obtaining necessary electronic protected health information during an emergency
4. Audit controls: implement hardware, software, and/or procedural mechanisms that record and examine activity in information systems that contain or use electronic protected health information
5. Integrity: implement policies and procedures to protect electronic protected health information from improper alteration or destruction
6. Person or entity authentication: implement procedures to verify that a person or entity seeking access to electronic protected health information is the one claimed
7. Transmission security: implement technical security measures to guard against unauthorized access to electronic protected health information that is being transmitted over an electronic communications network

Table 66.2 Addressable technical safeguards

1. Automatic log off: implement electronic procedures that terminate an electronic session after a predetermined time of inactivity
2. Encryption and decryption (developer): implement a mechanism to encrypt and decrypt electronic protected health information
3. Mechanism to authenticate electronic protected health information: implement electronic mechanisms to corroborate that electronic protected health information has not been altered or destroyed in an unauthorized manner
4. Integrity controls: implement policies and procedures to protect electronic protected health information from improper alteration or destruction
5. Encryption: implement a mechanism to encrypt electronic protected health information whenever deemed appropriate

outline the required and addressable technical safeguards required by the HIPAA Security Rule. Required elements need be implemented in all systems, while the decision to implement the addressable elements is made by the provider. If a provider chooses not to implement an addressable safeguard, he or she needs to document the reasons.

If you desire to create your own system to manage aspects of your practice, make sure you talk with your attorney about the regulations that must be followed.

HITECH Act

Subtitle D of the Health Information Technology for Economic and Clinical Health Act (HITECH Act) [5], enacted as part of the American Recovery and Reinvestment Act of 2009 [6], addresses the privacy and security concerns associated with the electronic transmission of health information. The American Recovery and Reinvestment Act of 2009 was signed into law on February 17, 2009, and established a tiered civil penalty structure for HIPAA violations. There are a number of aspects that affects ART laboratories. This summary will cover how the Act impacts the HIPAA Security Rules and Provider. Areas of interest are protecting ePHI and penalties for security violations.

Civil Penalties

The Act specifies penalties for violations that were eluded to in HIPAA (Table 66.3). A key clarification is that civil penalties can only be imposed in cases of willful neglect, provided the violation is corrected within 30 days.

Criminal Penalties

In June 2005, the US Department of Justice (DOJ) clarified who can be held criminally liable under HIPAA. Covered entities and specified individuals, whom "knowingly" obtain or disclose individually identifiable health information, face a fine of up to $50,000, as well as imprisonment up to 1 year. Offenses committed under false pretenses allow penalties to be increased to a $100,000 fine, with up to 5 years in prison. Finally, offenses committed with the intent to sell, transfer, or use individually identifiable health information for commercial advantage, personal gain, or malicious harm permit fines of $250,000 and imprisonment for up to 10 years.

Business associates are subject to the same civil and criminal penalties as covered entities for violating these requirements. This exposes technology vendors, practice management companies, transcription services, billing services, attorneys, accountants, and many other types of business associates to direct regulation under HIPAA. This goes into effect 1 year after the law's enactment (February 17, 2010).

Security Breach Notification Rules

As of September 23, 2009, covered entities are mandated to notify affected individuals of a breach involving "unsecured" PHI [7]. The law does not expressly mandate notification to affected individuals of any security breach. Individuals must be notified without "unreasonable delay" and in no event more than 60 days after discovery.

PHI and ePHI

ePHI stands for electronic protected health information. It is any protected health information (PHI) that is created, stored, transmitted, or received electronically. Protected health information (PHI) under HIPAA means any information that identifies an individual and relates to at least one of the following:

- The individual's past, present, or future physical or mental health
- The provision of health care to the individual
- The past, present, or future payment for health care

Table 66.3 Civil penalties

HIPAA violation	Minimum penalty	Maximum penalty
Individual did not know (and by exercising reasonable diligence would not have known) that they violated HIPAA	$100 per violation, with an annual maximum of $25,000 for repeat violations. Note: maximum that can be imposed by state attorneys general (regardless of the type of violation)	$50,000 per violation, with an annual maximum of $1.5 mm
HIPAA violation is due to reasonable cause and not due to willful neglect	$1,000 per violation, with an annual maximum of $100,000 for repeat violations	$50,000 per violation, with an annual maximum of $1.5 mm
HIPAA violations are due to willful neglect, but violations are corrected within the required time period	$10,000 per violation, with an annual maximum of $250,000 for repeat violations	$50,000 per violation, with an annual maximum of $1.5 mm
HIPAA violation is due to willful neglect and is not corrected	$50,000 per violation, with an annual maximum of $1.5 mm	$50,000 per violation, with an annual maximum of $1.5 mm

Table 66.4 Eighteen identifiers of protected health information

1.	Name
2.	Address (all geographic subdivisions smaller than state, including street address, city, county, ZIP code)
3.	All elements (except years) of dates related to an individual (including birth date, admission date, discharge date, date of death, and exact age if over 89)
4.	Telephone numbers
5.	FAX number
6.	E-mail address
7.	Social Security number
8.	Medical record number
9.	Health plan beneficiary number
10.	Account number
11.	Certificate/license number
12.	Any vehicle or other device serial number
13.	Device identifiers or serial numbers
14.	Web URL
15.	Internet Protocol (IP) address numbers
16.	Finger or voice prints
17.	Photographic images
18.	Any other characteristic that could uniquely identify the individual

Information is deemed to identify an individual if it includes either the individual's name or any other information that could enable someone to determine the individual's identity. Data are "individually identifiable" if they include any of the 18 identifiers of PHI (Table 66.4) for an individual or for the individual's employer or family member or if the provider or researcher is aware that the information could be used either alone or in combination with other information to identify an individual.

Instead of removing the data, sometimes, making the information more general is sufficient for deidentification, for example, replacing birth date with an age range.

Encryption of Data

The HITECH Act does not specifically require encryption. However, it clearly states, "Covered entities must comply with the requirements of the HIPAA Privacy and Security Rules by conducting risk analyses and implementing physical, administrative, and technical safeguards that each covered entity determines are reasonable and appropriate." So based on your risk analysis, you may or may not need to encrypt ePHI data. Data stored on devices that are mobile will be at high risk and should be encrypted. These might include:

- Laptop computers
- USB drives (thumb drives)
- Unsecured external hard drives
- Backup media
- Data transmitted via Internet

The HITECH Act defines two types of data with very different definitions and security concerns. These are data at rest and data in motion. Neither the HITECH Act nor the HIPAA regulations defines the type of encryption that should be used, but do mention to use current standards, which would be the encryption standards developed by the National Institute of Standards and Technology (NIST), (Special Publication 800–111; "Guide to Storage Encryption Technologies for End User Devices" Nov. 2007).

Data at Rest

Data at rest is data that resides in databases, file systems, and other structured storage methods. The three methods of securing and encrypting data at rest include full disk encryption, virtual disk and volume encryption, and file/folder encryption. The three methods offer different levels of encryption, the whole hard drive, a volume of a drive, or encryption only at the folder/file level. All three methods only offer protection while the data is at rest and requires the user authentication to decrypt the data.

Data in Motion

Data in motion is data that is moving through a network, including wireless transmission. All network traffic should be encrypted for both wired and wireless access. Many practices implement virtual private networks (VPNs) in order to connect multiple sites or allow users remote access to the office network. Since VPNs can be configured from many different protocols, it becomes complex to generalize about

its characteristics. A VPN provides an encrypted network tunnel between the client computer and the host the network.

Database Validation

Validation Basics

Data validation is the process of ensuring that a database solution operates on clean, correct, and useful data. It uses small programs, often referred to as scripts or routines, to create "validation rules" that check for correctness, meaningfulness, and security of data that are input to the system. The validation rules may be implemented either by comparing what is present in the database to a table of expected results often referred to as a data dictionary or by a program which steps through the data to check validation logic. The HIPAA Security Rule that governs these procedures is 164.312(c)(1) Integrity (Table 66.5). "The facility must implement policies and procedures to protect electronic protected health information from improper alteration or destruction."

This often encompasses authorization, validation, modification controls, and ensuring consistency of data. To facilitate tracking and problem resolution processes,

Table 66.5 IHS HIPAA security checklist

HIPAA Security Rule Reference	Safeguard (R) = Required, (A) = Addressable	Status Complete, n/a
Administrative Safeguards		
164.308(a)(1)(i)	**Security Management Process: Implement policies and procedures to prevent, detect, contain, and correct security violations.**	
164.308(a)(1)(ii)(A)	Has a Risk Analysis been completed IAW NIST Guidelines? (R)	
164.308(a)(1)(ii)(B)	Has the Risk Management process been completed IAW NIST Guidelines? (R)	
164.308(a)(1)(ii)(C)	Do you have formal sanctions against employees who fail to comply with security policies and procedures? (R)	
164.308(a)(1)(ii)(D)	Have you implemented procedures to regularly review records of IS activity such as audit logs, access reports, and security incident tracking? (R)	
164.308(a)(2)	**Assigned Security Responsibility: Identify the security official who is responsible for the development and implementation of the policies and procedures required by this subpart for the entity.**	COMPLETE
164.308(a)(3)(i)	**Workforce Security: Implement policies and procedures to ensure that all members of its workforce have appropriate access to EPHI, as provided under paragraph (a)(4) of this section, and to prevent those workforce members who do not have access under paragraph (a)(4) of this section from obtaining access to electronic protected health information (EPHI).**	
164.308(a)(3)(ii)(A)	Have you implemented procedures for the authorization and/or supervision of employees who work with EPHI or in locations where it might be accessed? (A)	
164.308(a)(3)(ii)(B)	Have you implemented procedures to determine that the Access of an employee to EPHI is appropriate? (A)	
164.308(a)(3)(ii)(C)	Have you implemented procedures for terminating access to EPHI when an employee leaves you organization or as required by paragraph (a)(3)(ii)(B) of this section? (A)	
164.308(a)(4)(i)	**Information Access Management: Implement policies and procedures for authorizing access to EPHI that are consistent with the applicable requirements of subpart E of this part.**	
164.308(a)(4)(ii)(A)	If you are a clearinghouse that is part of a larger organization, have you implemented policies and procedures to protect EPHI from the larger organization? (A)	
164.308(a)(4)(ii)(B)	Have you implemented policies and procedures for granting access to EPHI, for example, through access to a workstation, transaction, program, or process? (A)	
164.308(a)(4)(ii)(C)	Have you implemented policies and procedures that are based upon your access authorization policies, established, document, review, and modify a user's right of access to a workstation, transaction, program, or process? (A)	
164.308(a)(5)(i)	**Security Awareness and Training: Implement a security awareness and training program for all members of its workforce (including management).**	
164.308(a)(5)(ii)(A)	Do you provide periodic information security reminders? (A)	
164.308(a)(5)(ii)(B)	Do you have policies and procedures for guarding against, detecting, and reporting malicious software? (A)	

(continued)

Table 66.5 (continued)

HIPAA Security Rule Reference	Safeguard (R) = Required, (A) = Addressable	Status Complete, n/a
164.308(a)(5)(ii)(C)	Do you have procedures for monitoring login attempts and reporting discrepancies? (A)	
164.308(a)(5)(ii)(D)	Do you have procedures for creating, changing, and safeguarding passwords? (A)	
164.308(a)(6)(i)	**Security Incident Procedures: Implement policies and procedures to address security incidents.**	
164.308(a)(6)(ii)	Do you have procedures to identify and respond to suspected or know security incidents; mitigate to the extent practicable, harmful effects of known security incidents; and document incidents and their outcomes? (R)	
164.308(a)(7)(i)	**Contingency Plan: Establish (and implement as needed) policies and procedures for responding to an emergency or other occurrence (for example, fire, vandalism, system failure, and natural disaster) that damages systems that contain EPHI.**	
164.308(a)(7)(ii)(A)	Have you established and implemented procedures to create and maintain retrievable exact copies of EPHI? (R)	
164.308(a)(7)(ii)(B)	Have you established (and implemented as needed) procedures to restore any loss of EPHI data that is stored electronically? (R)	
164.308(a)(7)(ii)(C)	Have you established (and implemented as needed) procedures to enable continuation of critical business processes and for protection of EPHI while operating in the emergency mode? (R)	
164.308(a)(7)(ii)(D)	Have you implemented procedures for periodic testing and revision of contingency plans? (A)	
164.308(a)(7)(ii)(E)	Have you assessed the relative criticality of specific applications and data in support of other contingency plan components? (A)	
164.308(a)(8)	**Have you established a plan for periodic technical and non technical evaluation, based initially upon the standards implemented under this rule and subsequently, in response to environmental or operational changes affecting the security of EPHI, that establishes the extent to which an entity's security policies and procedures meet the requirements of this subpart? (R)**	
164.308(b)(1)	**Business Associate Contracts and Other Arrangements: A covered entity, in accordance with Sec. 164.306, may permit a business associate to create, receive, maintain, or transmit EPHI on the covered entity's behalf only of the covered entity obtains satisfactory assurances, in accordance with Sec. 164.314(a) that the business associate appropriately safeguard the information.**	
164.308(b)(4)	Have you established written contracts or other arrangements with your trading partners that documents satisfactory assurances required by paragraph (b)(1) of this section that meets the applicable requirements of Sec. 164.314(a)? (R)	
Physical Safeguards		
164.310(a)(1)	**Facility Access Controls: Implement policies and procedures to limit physical access to its electronic information systems and the facility or facilities in which they are housed, while ensuring that properly authorized access is allowed.**	
164.310(a)(2)(i)	Have you established (and implemented as needed) procedures that allow facility access in support of restoration of lost data under the disaster recovery plan and emergency mode operations plan in the event of an emergency? (A)	
164.310(a)(2)(ii)	Have you implemented policies and procedures to safeguard the facility and the equipment therein from unauthorized physical access, tampering, and theft? (A)	
164.310(a)(2)(iii)	Have you implemented procedures to control and validate a person's access to facilities based on their role or function, including visitor control, and control of access to software programs for testing and revision? (A)	
164.310(a)(2)(iv)	Have you implemented policies and procedures to document repairs and modifications to the physical components of a facility, which are related to security (for example, hardware, walls, doors, and locks)? (A)	
164.310(b)	**Have you implemented policies and procedures that specify the proper functions to be performed, the manner in which those functions are to be performed, and the physical attributes of the surroundings of a specific workstation or class of workstation that can access EPHI? (R)**	
164.310(c)	**Have you implemented physical safeguards for all workstations that access EPHI to restrict access to authorized users? (R)**	
164.310(d)(1)	**Device and Media Controls: Implement policies and procedures that govern the receipt and removal of hardware and electronic media that contain EPHI into and out of a facility, and the movement of these items within the facility.**	
164.310(d)(2)(i)	Have you implemented policies and procedures to address final disposition of EPHI, and/or hardware or electronic media on which it is stored? (R)	

(continued)

Table 66.5 (continued)

HIPAA Security Rule Reference	Safeguard (R) = Required, (A) = Addressable	Status Complete, n/a
164.310(d)(2)(ii)	Have you implemented procedures for removal of EPHI from electronic media before the media are available for reuse? (R)	
164.310(d)(2)(iii)	Do you maintain a record of the movements of hardware and electronic media and the person responsible for its movement? (A)	
164.310(d)(2)(iv)	Do you create a retrievable, exact copy of EPHI, when needed, before movement of equipment? (A)	
Technical Safeguards		
164.312(a)(1)	**Access Controls: Implement technical policies and procedures for electronic information systems that maintain EPHI to allow access only to those persons or software programs that have been granted access rights as specified in Sec. 164.308(a)(4).**	
164.312(a)(2)(i)	Have you assigned a unique name and/or number for identifying and tracking user identity? (R)	
164.312(a)(2)(ii)	Have you established (and implemented as needed) procedures for obtaining for obtaining necessary EPHI during and emergency? (R)	
164.312(a)(2)(iii)	Have you implemented procedures that terminate an electronic session after a predetermined time of inactivity? (A)	
164.312(a)(2)(iv)	Have you implemented a mechanism to encrypt and decrypt EPHI? (A)	
164.312(b)	**Have you implemented Audit Controls, hardware, software, and/or procedural mechanisms that record and examine activity in information systems that contain or use EPHI? (R)**	
164.312(c)(1)	**Integrity: Implement policies and procedures to protect EPHI from improper alteration or destruction.**	
164.312(c)(2)	Have you implemented electronic mechanisms to corroborate that EPHI has not been altered or destroyed in an unauthorized manner? (A)	
164.312(d)	**Have you implemented Person or Entity Authentication procedures to verify that a person or entity seeking access EPHI is the one claimed? (R)**	
164.312(e)(1)	**Transmission Security: Implement technical security measures to guard against unauthorized access to EPHI that is being transmitted over an electronic communications network.**	
164.312(e)(2)(i)	Have you implemented security measures to ensure that electronically transmitted EPHI is not improperly modified without detection until disposed of? (A)	
164.312(e)(2)(ii)	Have you implemented a mechanism to encrypt EPHI whenever deemed appropriate? (A)	

From http://www.hipaa.ihs.gov/documents/IHS_HIPAA_Security_Checklist.pdf. Accessed 4 December 2010

each interaction with the database must be assigned a unique sequence number or identifier, linking it back to the source:

- Authorization requires that the user must be properly authorized to interact with the database. The process that does this must be tracked and monitored.
- Input data validation requires that the data entered be checked by the system to ensure that it is valid and provides feedback to the user.
- Modification controls require that the system is able to ensure that the system does not have a significant risk of having undetected changes made to the database.
- Consistency of data requires that the system has in place a method to ensure that a user is identified with each data entry or modification of data. This is often done through a separate log file often maintained on a separate computer from which the actual database is located.

Validation Rules

Incorrect data validation can lead to data corruption or a security vulnerability. Data validation checks that data are valid, sensible, reasonable, and secure before they are processed. A validation rule is a criterion used in the process of data validation, carried out after the data has been entered into the database, and involves a validation program based on validation rules. This is to be distinguished from verification. Verification is one aspect of testing a product's fitness for purpose, whereas validation, for our purposes, ensures that our database operates on clean, correct, and useful data. Validation is the complementary aspect. Validation therefore ensures that the data entered is correct, whereas verification checks the integrity of the database.

System validation insures that the LIS manages information well, with the expected accuracy and reliability, file integrity, auditability, and management control [8]. Although

the vendor provides validation during development, it is the end user who is accountable for system validation [8]. Some vendors have voluntarily adopted the ISO 9000 standards of the International Organization for Standardization. However, the International Organization for Standardization is not a certifying body; if a vendor claims to follow its standards, the claim must be certified by a third party.

End-user validation usually involves several steps. An organized approach to validation of a database solution by the end user is presented in detail by Cowan et al. [8]. They describe a five-step process:

1. Identification and description of the system to be validated. To address these issues, the intended function of the system must be specified and documented.
2. Specification of the stage in the system life cycle. A database can be thought of as having a life cycle consisting of several stages. It will be bought, used, upgraded, and perhaps eventually replaced. Validation tasks depend on the cycle stage of the system. These include a definition of requirements phase, a system design specification phase, an implementation phase, a test phase, a checkout phase, and an operation and maintenance phase. Specifying the stage in the system life cycle allows testing and validation to be conducted at distinct points of development of the information system.
3. Development of hazard analyses. Hazard analysis is determination of the potential degree of human harm that might result from the failure of a device or system (i.e., specification of the penalty for failure). This may be as serious as issuing bad data, or it may be minor but annoying, such as printing poor-quality paper reports.
4. Identification of regulatory concerns. This will determine which regulatory agency, if any, will claim jurisdiction and which set of standards will apply to the operation of the system and the validation process. The agencies may include the FDA, state health departments or licensing bodies, and professional accrediting organizations, such as the College of American Pathologists and the Joint Commission on Accreditation of Healthcare Organizations.
5. Documentation. Validation of a system produces a large volume of documentation. These documents include not only the general statements about validation and test plans but also the test scripts and attached listings, screen prints, and test reports, which must include an explicit statement of pass-fail status, the signature of the person conducting the test, and name of the person reviewing the test status and disposition. This documentation must be compiled, organized, and kept in a designated location for management and regulatory review.

Validation is a continual process that needs to be documented in the SOP manual for the laboratory ensuring that a database solution operates on clean, correct, and useful data.

Conclusions

Software systems implemented into an ART laboratory need to be assessed very carefully and deliberately to make sure it not only meets your business goals but also meets regulatory and reporting requirements.

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Regulation, Licensing, and Accreditation of the ART Laboratory

67

Doris Baker

Abstract

Assisted reproductive technology (ART) laboratories are associated with assisted reproductive medical practices. These entities are unique in that they do not perform laboratory testing for purposes of diagnosis and treatment of disease but work with gametes and preembryos, performing interventions and treatments for infertility. These interventions and treatments vary among ART practices and extend from basic in vitro fertilization to highly complex and multifaceted genetic and molecular procedures. The level of education, training, and experience of the embryologists performing the work diverges as well, ranging from doctoral scientists specifically educated for the field to individuals trained on-site to perform procedures. Oversight of embryology and associated personnel may be by statute, regulation, guidelines, accreditation, licensure, or certification, or the field of embryology and embryologists may function without benefit of any type of scrutiny. Insufficient scrutiny, or inconsistent and confusing regulation of these ART interventions and treatments, as well as qualifications and oversight of the individuals performing the tasks, is of general concern. The procedures, which are often controversial, affect not only the infertile couple and any offspring that might result but also in cases of third-party reproduction multiple individuals who are involved, and because of associated medical, economic, scientific, ethical, religious, and legislative issues, ART has an effect on society as a whole. As a result, there is a movement to bring formal recognition and professional status to embryology. Members of the profession will then be able to petition for certification as an expectation for practice. Once embryology board certification is the standard, it will become a regulatory requirement, conferring embryologists parity with other medical and healthcare professionals. As well, members of the profession will be able to promote standardization for the field, have input into guidelines and development of quality standards for "laboratory" accreditation, and move toward self-regulation.

Keywords

Accreditation • Assisted reproductive technology • ART interventions • ART laboratory • Certification • Directive • Embryologist • Embryology • Guideline • Law • Legal • License • Licensing body • Mandate • Oversight • Standard • Statute

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The World Collaborative Report on Assisted Reproductive Technology, 2002 [1, 2] from the International Committee for Monitoring Assisted Reproductive Technology (ICMART) analyzed assisted reproductive technology (ART) practices using data from 1,563 clinics in 53 countries for the year 2002 and estimated that between 219,000 and 246,000 babies were born worldwide through ART from the more than 600,000 cycles that were initiated. Due to reporting and methodological problems, these numbers are estimated to represent 60–70% of ART activity worldwide. The approximation for the number of births resulting from ART procedures in 2002 revealed an increase over the previous report from 2000, which estimated that approximately 197,000 to 220,000 babies resulted from ART procedures [3]. The ICMART 2002 report, which was the Eighth World Report on assisted reproductive technologies, suggests that ART activity continues to show a general increase over time [1, 2].

ART procedures are performed in specialized centers in developed nations of the world, although there is variation in the use of these services. Lancaster reported that countries with the highest treatment cycles were Denmark, Finland, and Australia and those with the lowest treatment cycles were the United States, New Zealand, and the United Kingdom. Other European countries had intermediate treatment ratios [4].

ART Facilities

Facilities that are referred to as ART or embryology *laboratories* are associated with assisted reproductive medical practices, although the term *laboratory*, by definition, is not consistent with the services provided by these facilities. A laboratory is defined as a place for doing tests and research procedures and for preparing chemicals [5] (Table 67.1). A clinical laboratory is for examination of materials derived from the human body for the purpose of providing information on diagnosis, prevention, or treatment of disease [6].

Table 67.1 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 Bioanalysts—evaluates, through the certification process, individuals who wish to enter, continue, or advance in the clinical laboratory profession
American Board of Bioanalysts 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 and for establishing standards for embryology in the United States
American Society for Reproductive Medicine (ASRM)—professional organization devoted to advancing knowledge and expertise in reproductive medicine and biology
Assisted hatching—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 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 and embryo transfer, gamete intrafallopian transfer, zygote intrafallopian transfer, tubal embryo transfer, gamete and embryo cryopreservation, oocyte and embryo donation, gestational surrogacy, do 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 UK
Board certified—having completed the process of board certification in a specialty field
Board examination—qualifying examination for 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 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 is regulated as a human cell, tissue, and cellular and tissue-based product or HCT/P; includes reproductive tissues
Centers for Control and Prevention (CDC)—one of the major operating components of the Department of Health and Human Services 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 & Medicaid Services (CMS)—federal agency within the United States 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, 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

(continued)

Table 67.1 (continued)

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 of, of 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 CMS; 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 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, sets standards of quality and safety for the donation, procurement, testing, processing, preservation, storage, and distribution of human tissues and cells
Embryo—state of 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
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 Bioanalysts. Minimum requirements include a B.S. degree, having personally performed at least 60 ART procedures in humans under current standards of care, pass ABB's examination for ELD, and have 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; 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; 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 in which embryos created by the intended parents are transferred to the surrogate's uterus, which has been hormonally prepared to carry a pregnancy with the agreement that she will give the offspring to the genetic parents
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 United States government's principal agency for protecting the health of 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 regulates 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 use of gametes and embryos in fertility treatment and research
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
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
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
Oversight—supervision, control, overseeing, managing, administration
Preembryo—conceptus during early cleavage stages of development until approximately 14 days postfertilization

(continued)

Table 67.1 (continued)

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
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, to report pregnancy success rates and report each embryo laboratory used by the program and whether it is certified—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)—transfer 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; 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—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
Traditional surrogacy—treatment in which a woman is inseminated with sperm for the purpose of conceiving for an intended recipient; 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
Zygote—one-cell stage that follows breakdown of the pronuclear membrane and precedes the first cleavage
Zygote intrafallopian transfer (ZIFT)—procedure in which zygotes are transferred into the Fallopian tube

The United States Clinical Laboratory Improvement Amendments (CLIA) defines a laboratory as "…a facility for the biological, microbiological, serological, chemical, immuno-hematological, hematological, biophysical, cytological, pathological, or other examination of materials derived from the human body for the purpose of providing information for the diagnosis, prevention, or treatment of any disease or impairment of, or the assessment of the health of human beings" [7]. ART embryology facilities provide highly technical and scientifically advanced services that are actually interventions and/or treatments and are therefore not consistent with approved procedures performed in clinical laboratories for diagnosis, prevention, or treatment of disease. These interventions and/or treatments are performed by embryologists, who have varying levels of education, experience, and expertise [8].

ART Interventions/Services

As with usage of ART for treatment of infertility, 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 [9, 10]. The commonality is that the services involve the manipulation of both gametes with the goal of creating human life in an artificial setting.

Interventions range from the basic in vitro fertilization (IVF) and preparation for embryo transfer (ET) that resulted in the first in vitro birth in the United Kingdom in 1978 [11] to very advanced technical and molecular procedures and treatments [9]. Some laboratories offer services that are spin-offs from IVF such as gamete intrafallopian transfer (GIFT) and zygote intrafallopian transfer (ZIFT), although GIFT, which requires laparoscopy and is therefore more complicated than IVF, appears to be limited to niche populations that may reflect religious dogma opposed to fertilization outside the body, or to regulation [9]. Embryo culture may be restricted to a cleavage stage or extended to the blastocyst stage of development [12]. 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 sperm is needed for insemination or may be expanded to include cryopreservation of zygotes, blastocysts, oocytes [13], donor sperm, and ovarian and testicular tissues [14, 15]. Micromanipulation

procedures typically are routine as well and generally include intracytoplasmic sperm injection (ICSI) [16, 17], and perhaps assisted hatching (AH) [18]. Embryo biopsy for preimplantation genetic screening and diagnosis (PGS and PGD) [19], which requires considerable technical skill, is available in some ART laboratories as is the complex process of maturation of immature oocytes [20, 21].Third-party reproduction, which also requires embryology expertise, may be practiced as well, including use of donor sperm, donor oocytes, and donor embryos, and utilization of gestational carriers and/or surrogates [22]. In addition to clinical manipulations, some embryologists may contribute to research that ranges from data collection of clinical outcomes to investigations that encompass oocyte cytoplasmic and/or nuclear transfer [23, 24], somatic cell nuclear transfer (SCNT) [25], and experimentation on the preembryo [9, 26].

Concerns Regarding ART Laboratory Interventions

Lack of oversight or insufficient scrutiny of these ART interventions and treatments, as well as qualifications and oversight of the individuals performing the tasks, is of general concern. These often controversial procedures not only affect the infertile couple and any offspring that might result, but in cases of third-party reproduction multiple individuals are involved, and because of associated medical, economic, scientific, ethical, religious, and legislative issues, ART has an effect on society as a whole [8]. Scientists and the medical community are concerned about the safety of certain treatments since long-term outcome studies are not yet available. For example, reports suggest there is an increased risk of birth defects, especially those due to imprinting disorders in children conceived by ART, particularly if ICSI is an intervention [17, 27–37]. Geneticists are apprehensive regarding procedures that result in offspring having DNA from two maternal sources, mitochrondial DNA from cytoplasmic transfer [23], or nuclear DNA from oocyte nuclear transfer [24], as well as genetic considerations related to ICSI [30], and for the potential development of chimeras from fusion of two embryos developing in vitro [38]. Consanguinity is an issue with third-party reproduction [39, 40]. This is of particular concern in countries without a tracking system for donated gametes and embryos [41]. As noted, many religious groups object to ART procedures [42, 43]. This is highlighted in countries that are predominantly Catholic. The Roman Catholic Church is opposed to ART because procedures separate procreation from normal sexual function [42]. ART is practiced in Italy but is restricted to couples in a stable relationship, and cryopreservation of oocytes, sperm, oocyte and embryo donation, surrogacy, PGD, and experimentation on the preembryo are proscribed by statute [9]. ART cannot be practiced in the predominately Catholic country of Costa Rica and, in fact, violates the constitutional law that defines the beginning of personhood at the time of fertilization [44]. Ethicists and others are concerned about misuse of the technology, including the potential for reproductive cloning [26, 45–50]. PGD is a concern if used for sex selection [51, 52], especially since the male gender is more highly valued in certain cultures [53–55]. Societies with a history of preferred ethnicity are also concerned. This explains why sperm and oocyte donation for IVF, surrogacy, and experimentation on the preembryo are not allowed by law in Austria and Germany, and PGD is prohibited in Germany [9]. Society as a whole has many concerns including equity in access to ART procedures in some countries, including the United States [9, 56], impact on family values associated with third-party reproduction, including gamete and embryo donation and use of gestational carriers; [22, 57–59] and increases in overall costs of health care associated with the care of mothers and babies in higher-order multiple pregnancies [60–62] and care of older mothers in pregnancies that could only be established by means of ARTs [61, 62]. Disquiet associated with ART practices, including the embryology setting, burgeons when research is performed on the preembryo [25, 49, 50, 63, 64] or when embryos are developed for research [65]. Tantamount or paramount to embryo research is embryo reduction in multiple pregnancies [66]. Furthermore, legal issues may result from any ART intervention or treatment [67, 68].

Although the physician and patient decide on a course of treatment, it is the embryologist who is responsible for performing these interventions and treatments that command advanced knowledge, highly developed technical skills, and absolute attention to detail; attributes that are crucial for positive outcomes and prevention of errors [8]. Mix-ups that have resulted in insemination with the wrong sperm and the subsequent birth of offspring to the wrong couple have occurred in the embryology setting [69–71]. As well, deliberate physician misuse of embryos that have occurred required interaction with embryology and embryology personnel [72–74]. Basic ART interventions, including culture methods, ICSI, and assisted hatching, may be responsible for increasing rates of monozygotic twinning [75–77]. Increase in epigenetic-related birth defects associated with ICSI is another embryologist concern since it has not been determined if this increase is due to the infertility problem requiring the intervention, the methodology, or both [29, 30]. The embryologist also has a role in interventions that impact the serious issue of higher-order multiple births. Whether culture is restricted to cleavage-stage embryos or extended to the blastocyst determines the number of embryos for transfer, and it is the skill level of the embryologist that determines whether culture to this more advanced stage is successful [78]. Quality of the embryos, regardless of developmental stage, influences the actual number the physician elects to transfer [62]. Again, the knowledge and skill level

of the embryologist plays a key role in culturing quality embryos. When too many embryos are transferred due to stage of development or questionable quality, a higher-order multiple pregnancy may result [61]. Higher-order multiple pregnancies may then lead to fetal reduction [66], which is a procedure that may negatively impact the ongoing pregnancy as well as raise religious and ethical concerns. Third-party reproduction is another concern. Although utilization of donor gametes and oocytes is a physician-patient decision, the embryologist is responsible for maintaining standard operating procedures, including proper documentation [79, 80]. Donor anonymity status is determined by law or guideline [9], but it is a responsibility of the embryologist to comply with this directive [79, 80].

Regulation, Licensure, Accreditation, and Guidelines

In spite of the complexity of procedures and associated work performed by embryologists, oversight or regulation of ART laboratories varies and, when addressed, is often included under the umbrella of the overall ART medical practice [9]. Oversight of embryology settings and embryologists may be by regulation, statute, guidelines, licensure, or certification, or the field of embryology and embryologists may function without benefit of any type of scrutiny [9]. A *regulation* is an authoritative rule dealing with details or procedures that is issued by an executive authority or regulatory agency of a government (i.e., the United States Food and Drug Administration or FDA has regulatory authority over donor and recipient reproductive tissues [79, 80] as does Directive 2004/23/EC of the European Parliament and of the Council [81]), whereas a *statute* or *law* is enacted by legislature. The Human Fertilisation and Embryology Act of 1990 [82] that regulates ART practices in the United Kingdom was the first statutory body of its type in the world. A *guideline* is a principle put forward to set standards or determine a course of action. Guidelines for ART are typically set by a professional society (i.e., the American Society for Reproductive Medicine ASRM (www.ASRM.org) in the United States and the European Society of Human Reproduction and Embryology (ESHRE) (www.ESHRE.org) in Europe), but may be established by a government agency. 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 violation of guidelines [9].

Countries operating under statute may or may not have surveillance and may or may not have a *licensing body*, which is a competent authority able to grant permission to exercise a certain privilege that, without such authorization, would constitute an illegal act. The most recent survey of ART and ART laboratory practices is the International Federation of Fertility Societies Surveillance 07 (IFFS 07) [9].IFFS 07 data, which were based on responses from 57 developed countries, reported that 29 countries or sovereign states operated under statutes, 18 under guidelines, and 10 without either statues or guidelines. Twenty-one of the 29 nations with legislation had a licensing body and performed clinical surveillance, either in the form of a periodic report, on-site inspection, or both. Laboratory surveillance was performed in different ways, and 14 countries had laboratory accreditation. *Accreditation* gives official authorization or approval by providing credentials that vouch for conforming to a standard. Accreditation typically involves an entire process, including credentials for those responsible for the standard. Accreditation is an expectation for specialized institutions (medical schools) or for professional practice. Personnel requirements for embryology laboratory accreditation are not included in the IFFS 07 report, but many include a requirement for *certification*, defined as the guarantee to meet a *standard* with standard defined as a basis for comparison or a reference against which other things can be evaluated.

In countries with statutes, penalties for violations differed and ranged from fines to loss of license and, in the most severe cases, imprisonment. There were penalties for violations of statutes with regard to laboratory procedures in 19 of the 29 countries with legislation [9].

Like countries with statutes, surveillance may be performed, and periodic reports required under guidelines. Ten of the 15 countries with guidelines had clinical surveillance, and there is embryological laboratory surveillance performed in eight of those countries [9].

Embryology "Laboratory" Oversight: A Comparison

Comparing a country with specific laws for ART, the United Kingdom (UK), with the United States (USA), a country operating without an overall federal statute that is specific for assisted reproduction, is illustrative of how ART, including the embryology facility, actually must respond to a number of oversight entities. In the UK, the Human Fertilisation and Embryology Act of 1990 is a statute for regulation of ART [82]. It is a departmental public body accountable to the Department of Public Health. Under this Act, the Human Fertilisation and Embryology Authority (HFEA) (http://www.hfea.gov.uk/) was established. HFEA is charged with regularly inspecting fertility clinics and research centers to ensure that every licensed clinic or center is adhering to standard safety and ethical rules set out by the UK government. In order to issue or renew a license, the clinic's premises and

facilities, including laboratory processes and services offered, are inspected as well as the suitability of the person responsible and the staff providing the services [83]. HFEA has adopted guidelines for good laboratory practice developed by the Association of Clinical Embryologists' Laboratory Accreditation Committee (http://www.embryologists.org.uk/), a professional organization for embryologists. Under statute, HFEA has the authority to issue sanctions for violations, with the penalty commensurate with the violation, and which may include revocation of license that prevents continuation of practice or closing a center.

In addition to HFEA, the UK is regulated by Directive 2004/31/EC of the European Parliament and of the Council for setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage, and distribution of human tissues and cells [81]. The UK responds to practice guidelines as well. The professional organization, the ESHRE (http://www.eshre.eu/01/default.aspx?pageid=3), has input into the ART practices by disseminating information, providing training and education, maintaining databases, and implementing methods to improve quality assurance and safety in the laboratory.

The United States does not have a federal statutory body that governs the overall practice of ART and embryology and is therefore considered to operate under guidelines [9]. The ASRM (http://www.ASRM.org.), the primary professional society for ART, issues practice guidelines that include guidelines for human embryology and andrology laboratories [84]. Although there is not a specific federal legislative body for regulating ART and embryology, there is regulation. The United States FDA under 21CFR Parts 1270 and 1271 [79, 80] determines eligibility for donors of human cells, tissues, and cellular and tissue-based products, to prevent the introduction, transmission, or spread of communicable diseases, and establishes current good tissue practices. Reproductive tissues, including semen, oocytes, and embryos, must meet the FDA requirements for screening and testing for infectious disease, quarantine, storage, product labeling, and record keeping. Andrology testing is regulated by CLIA [7], within the Centers for Medicare & Medicaid Services (CMS) within the US Department of Health and Human Services (HHS) (http://www.hhs.gov/), which has regulatory authority, making embryology accountable to this entity if sperm concentration is determined in the embryology facility prior to oocyte insemination. Public Law 102-493, the Fertility Clinic Success Rate and Certification Act of 1992 [85], was enacted in 1999 [86] to provide for accurate reporting of ART success rates and for the development of a model embryology laboratory. The HHS secretary, through the Centers for Disease Control and Prevention (CDC), annually publishes pregnancy success rates as reported by ART programs as well as ART clinics failing to report data (http://www.cdc.gov/art/ARTReports.htm).

The model certification program details definitions, administrative requirements, and embryo laboratory standards, including procedures performed and personnel qualifications [86]. Public Law 102-493 was not intended to provide federal oversight, leaving adoption and implementation at the discretion of each individual state. Individual states would be responsible for administration of the certification program with provisions for inspection and certification of embryo laboratories by States, or by approved accreditation organizations for states not adopting the law. It is noteworthy that this law provided that a state would not establish any regulation, standard, or requirement, which had the effect of exercising supervision or control over the practice of medicine in ART programs of clinics. To date, there is no documentation that ART clinics not reporting success rates to the CDC have received sanctions or that any state has adopted the certification program. Most ART laboratories are accredited by The College of American Pathologists (www.CAP.org) via the College's Reproductive Laboratory Accreditation Program, a peer-review process that is based on a checklist for three areas: general laboratory, andrology, and embryology. Items evaluated during on-site inspections are based on the individual laboratory's test/procedure menu (http://www.cap.org/apps/docs/laboratory_accreditation/masterActivityReports/master_activity_menu_pt_options_rlap.pdf). Although states have not adopted the Public Law 102-493 certification program, embryology must comply with all new and existing state laws. For example, in the state of Louisiana, frozen embryos have individual rights as defined by a 1986 state statute, and surrogacy is outlawed in some states [87–89].

Whether operating under statute, regulation, guidelines, or a combination of laws and guidelines, oversight of ART tends to be complex and lacking coherence. Furthermore, intent of legislation and guidelines is not necessarily realized, as demonstrated by lack of penalty for nonreporting to the CDC and states not adopting the Laboratory Certification Program. The role of the embryologist and qualifications for the position are often not clearly defined or are inconsistent among regulations, guidelines, and certification and accreditation processes. In the UK, the HFEA Code of Practice (http://www.hfea.gov.uk/code.html) describes the inspection process and denotes that the inspection evaluates the clinic's premises and facilities, laboratory processes, and the ability of the clinic to provide the services [83]. Embryologists in the UK, as well as other European countries, must comply with Directive 2004/23/EC of the European Parliament and of the Council that sets standards for donors and recipients of reproductive tissues. Regarding personnel, this directive simply states that personnel directly involved with the donation, procurement, testing, processing, preservation, storage, and distribution of these tissues and cells should be "appropriately qualified and provided with timely and relevant training" [81].

In the USA, Public Law 102-493 defines qualifications for an embryology laboratory director (ELD), which includes being a physician or doctoral scientist with experience in ART, and for the embryology supervisor, which includes holding a minimum of a master's degree in science and documented experience in ART. Public Law 102-493 allows that testing personnel can only perform procedures authorized by the director and for which training has been documented [85]. The College of American Pathologists Reproductive Laboratory Accreditation Program (CAP-RLAP) outlines qualifications for the ART laboratory director and other personnel in standard I [90]. The standard states that the laboratory director shall be a qualified, board-certified pathologist, other qualified physician, or a doctoral scientist with the exception that a bachelors- or masters-prepared scientist may serve as the ART laboratory director, provided he/she has been certified as an ELD by the American Board of Bioanalysis (ABB) (http://www.aab.org/aab/Certifications_Qualifications.asp?SnID=963342779). Qualifications for testing personnel are not defined. Instead, the director must "ensure that there are sufficient qualified personnel with adequate documented training and experience to supervise and perform the work of the laboratory." This is in general agreement with the ASRM guidelines for embryology laboratory director and supervisor [84]. However, the ASRM guideline addresses the embryology laboratory technologist and recommends that qualifications include an earned bachelor's or master's degree in science from an accredited institution, documented embryology training, and continuing education. The FDA denotes under the quality program section for inspection of human cells, tissues, and cellular and tissue-based products (CGTPs), which includes reproductive tissues, that "a program should exist that insures that all personnel involved in activities that relate to the core CGTPs are properly trained and educated to perform their job" [80].

Until 2009, the only education leading to certification for embryologists in the UK was provided by the Association of Clinical Embryologists. This professional group administers a Certificate in Clinical Embryology (http://www.embryologists.org.uk/education/), which is a practical-based qualification appropriate for degree entrants employed as in embryology as clinical scientists. A suitably experienced "in-house" embryologist is necessary to act as a supervisor, and a member of the Association of Clinical Embryologists Training Committee acts as an assessor providing overall guidance. The training consists of a logbook subdivided into modules that documents the candidate's performance of the various practical procedures used in ART along with didactic testing. The program takes approximately 2 years to complete. The certificate provides parity with other clinical scientists in the National Health System (NHS) and is considered an essential requirement when applying for state registration. In the past, the Association of Clinical Embryologists administered a Diploma in Clinical Embryology, which was a theory-based qualification for experienced embryologists that required a research project and a final examination set from an extensive syllabus. This option is no longer available, and the Association of Clinical Embryologists Professional Development Committee now aims to develop both a program for continuing education and a senior qualification for clinical embryologists.

Until 2010, the only certification available for embryology laboratory personnel in the USA has been administered by the ABB (http://www.aab.org/aab/Certifications_Qualifications.asp?SnID=920409100). The ABB certifies high-complexity laboratory directors, embryology laboratory directors, clinical consultants, technical supervisors, and general supervisors based on education, experience, and successfully passing a written examination.

Recent Developments

Due to the variation in oversight of embryology, the differing expectations for qualifications for embryologists, and increased demands for scientific knowledge and advanced skills to meet the continually advancing field, there is a focus on acknowledging embryology as a profession, developing formal recognition of embryologists as professionals, and establishing universal standards for the discipline. In the past, absent professional training and curricular-based education for the field, embryologists were trained on-site by those already established in ART, creating a situation where, if the instructor was unqualified or ineffective, bad techniques and false premises would be perpetuated [8]. There have been significant advances in education in embryology with several university degree programs now available worldwide in addition to certificates, diplomas, and specialty courses in ART and embryology. In IVF.net lists, 37 such programs (http://www.ivf.net/ivf/embryology_courses-b348.html) and additional curricular-based programs have since been added [91]. Availability and types of education in the field correspond to the demand for in-depth scientific knowledge necessary to keep pace with the ever-evolving field that now incorporates molecular biology, genetics [92], proteomics [93], genomics [94, 95], stem cell research [26, 96], and new cryobiology methods that are a response to the increasing types of cells and tissues that can be successfully frozen and thawed [14, 15]. In line with the advanced technology is the ever-increasing need to understand legislation and regulation that are a reaction to the new technologies and interventions that are now included in embryology [87, 97, 98].

The embryology professional organization in Europe, ESHRE, has taken the lead in certifying competent embryologists and developing a formal recognition for these professionals. ESHRE has devised a system that provides two levels of certification (http://www.eshre.eu/accreditation_and_certification/page.aspx/17) based on three different tracks: (1) an initial fast track for senior clinical embryologists with a laboratory

director position and minimum of 10 years experience in human ART; (2) a regular track for senior-level clinical embryologists; and (3) a regular track for clinical embryologists. Certification for each track is based on academic credentials (minimum M.Sc. in Natural Sciences for senior-level track and minimum of B.Sc. in Natural Sciences for clinical track), a logbook that documents required hands-on experience in an ART laboratory, and successfully passing a comprehensive, stringent examination based on an in-depth curriculum that includes cell and molecular biology, genetics, male and female reproduction, fertilization, embryo development and early pregnancy, infertility reasons, work-ups and treatments, laboratory procedures including cryopreservation, quality assessment, statistics, handling data, ethics, and legislation. As part of their mission, ESHRE members are dealing with standardization for practice.

The newly developed (2009) American College of Embryology (http://www.embcol.org/) is also certifying embryologists based on an in-depth body of knowledge that is very similar to the ESHRE model, but with more rigorous academic requirements for qualifying for certification. American College of Embryology offers four levels of certification for practicing embryologists: Embryologist (EMB Level IV) requires a Ph.D. in science or an M.D., hands-on ART experience, successfully passing the American College of Embryology board examination, and having successfully passed a skills proficiency examination in at least one of the following areas (ICSI, embryo biopsy, cryopreservation). The embryology associate (EMA Level III) requires a minimum of an M.S. degree in science, hands-on ART experience, successfully passing the ACE board examination, and having successfully passed a skills proficiency examination in at least one area (ICSI, embryo biopsy, cryopreservation). The embryology technologist (EMT Level II) requires a B.S. degree in science or medical laboratory science, hands-on ART experience, successfully passing the American College of Embryology board examination, and having successfully passed a skills proficiency examination in at least one of the specified areas. The graduate embryologist (Level I EMG) must hold an earned degree Ph.D., M.S., or B.S. in science plus a graduate certificate in Embryology or Reproductive Laboratory Science from an accredited university or hold a M.S. degree in Embryology or Reproductive Laboratory Science from an accredited university, and successfully pass the American College of Embryology theoretical examination. The Level IV EMG is qualified for an entry level position in ART. American College of Embryology also recognizes professionals who contribute to the discipline but are not practicing embryologists by providing the embryologist nonclinical track, which requires a Ph.D. in life sciences or an M.D. from an accredited university, and successfully passing the American College of Embryology board examination. Those holding certification in the nonclinical track cannot practice in embryology without completing all qualifications for Level IV.

For all levels of certification, American College of Embryology requires continuing education (CE) units in order to renew certification. To ensure that embryologists remain current in all aspects of the field of embryology, recertification will be required every 5 years and will be dependent on submission of an acceptable case log and reexamination. American College of Embryology is committed to providing this high level of quality to promote professional status for embryology and embryologists and to prepare the field to move toward self-regulation.

ACE, the only self-governed organization of embryologists in the United States, holds the position that embryology needs to be independently regulated because the field is unique and because currently oversight is either lacking or inappropriate. This state exists due to lack of consensus regarding the status of the human embryo. Whether the embryo is afforded human status varies among nations and among religious factions. Differences in the recognized time during human development, after which a human person is considered to exist, range from the time of fertilization to the time of delivery [7]. Lack of agreement on the status of the embryo has impacted and continues to impact regulation of embryology. If the embryo is considered human, then the embryology laboratory is providing a medical care, and therefore, embryology has to be regulated as a medical profession. On the other hand, if the embryo is not human, it cannot be regulated by medical boards. Past efforts to include embryology laboratories under CLIA [99] have failed because of the definition of facilities and testing that are under the CLIA mandate, specifically that the CLIA directive covers "materials derived from the human body," [7] are not applicable to embryos. While spermatozoa and oocytes would fall under this definition, and could therefore be regulated under the CLIA edict, the embryo created by IVF represents a new organism that did not originate from the human body and technically is not subject to CLIA regulation. The status of the human embryo and associated regulation is a major social, religious, policy, ethical, legal, and political issue and one that is not likely to be resolved in the near future, if ever.

American College of Embryology maintains the position that the human embryo should not be considered as either human or simply tissue, but that it has a unique place during human development and is a separate entity that must be fully recognized and respected. American College of Embryology further believes that embryology should be regulated by embryologists, similar to medical boards regulated by physicians, to ensure that human embryos are cared for by well-trained professionals who are not only scientifically capable but also indoctrinated to uphold the respect accorded these unique entities, much as medical students must respect the once living cadaver. Status of the human embryo also impacts regulation of PGS/PGD, which varies among countries [100]. Conflicting opinions regarding the moral acceptability of manipulating embryos during PDS/PGD also influence attitudes

toward regulating these procedures in the USA [101, 102]. American College of Embryology promotes the inclusion of PGS/PGD under embryology regulation since as with embryos, biopsied blastomeres removed for PGS/PGD are not derived from the human body and therefore are not under the CLIA mandate, nor based on the American College of Embryology position regarding embryo status, should they fall under the auspices of medicine.

Efforts undertaken by ESHRE in Europe and development of American College of Embryology in the United States are important steps in moving embryology toward professional status. Both professional organizations provide education and certification for qualified embryologists on an international basis, which will facilitate standardization of practice in embryology and prepare embryologists for self-regulation.

Future

As embryology becomes recognized as a profession and the embryologist as a professional, which must happen as the field continues to grow and interventions require greater knowledge of the sciences as well as advanced skills, regulation, licensing, certification, and accreditation will be influenced. Once embryology is viewed as an established profession, current and anticipated needs can be addressed under one umbrella. The first step, ensuring that individuals are well educated and trained at a level to perform current interventions and treatments, as documented by certification based on stringent criteria that include skills proficiency testing, has already been undertaken and must be maintained. Examinations must be based on a constantly evolving body of knowledge, and continuing education must be developed that is readily available (e.g., web-based) and affordable for all levels of embryologists. Recertification based on examination and continued practice in ART will be mandatory in order for embryologists to continue to meet the needs of their complex and ever-evolving profession. Members of the profession will then be able to petition for certification as an expectation for practice. Once embryology board certification is the standard, it will become a regulatory requirement, conferring embryologists' parity with other medical and healthcare professionals. As well, members of the profession will be able to promote standardization for the field and have input into guidelines and development of quality standards for "laboratory" accreditation. Embryologists also will be able to lobby for terminology that accurately describes the profession. For example, the term "test" should be descriptive of a laboratory procedure that yields a result and that is associated with quality control for the assay, such as the determination of sperm concentration. Most embryology procedures, on the other hand, should be referred to as treatments or interventions. The misnomer, "embryology laboratory" could be corrected and renamed to accurately reflect the services provided, such as "embryology facility" or "embryology services." With professional recognition and board certification accepted as a standard for the professional, embryologists will be able to move toward self-regulation for the field.

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Legislation in the United Kingdom

68

Rachel Cutting

Abstract

In this chapter, we review the legislative and regulatory framework concerning assisted reproductive technologies in the United Kingdom. The HFEA currently provides a comprehensive regulatory mechanism that ensures patient protection. Mechanisms are available either to place conditions on center's license or withdraw a license if noncompliance against the legislation is demonstrated. The regulatory framework ensures high standard of practice and ensures research is ethically acceptable within our society. Although seen by some as over bureaucratic, the system has been widely emulated in other countries and reassures the public that this sensitive and ethically challenging area of medical practice is performed to the highest standards.

Keywords

ART legislation in the United Kingdom • In vitro legislation in the UK • Human Fertilization and Embryology Authority • Licensing of ART laboratories in the UK • Ethics in the ART laboratory

The accumulation of many years of research with both human and animal embryos resulted in the successful birth of the world's first IVF baby in 1978 [1]. Although, this was a considerable breakthrough in the treatment of the infertile couple it raised both public and professional concerns regarding the ethical and moral boundaries around producing embryos in the laboratory.

Early Years of Regulation

In order to shape future legislation in 1982, a committee of inquiry into human fertilization and embryology was formed with Mary Warnock as the chair. The specific terms of reference were "to consider recent and potential developments in medicine and science related to human fertilization 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 scope included all processes involved in in vitro *fertilization* including donor insemination and surrogacy. The comprehensive report which was published in 1994 made over 60 recommendations and encompassed all aspects of treatment provision including [2]:

- The scope and organization of services
- The techniques for the alleviation of infertility
- Wider use of techniques
- The freezing and storage of human semen, eggs, and embryos
- Scientific issues
- Possible future developments in research
- The regulation of infertility services and research

The report recognized that the human embryo should be protected by law and unauthorized use should result in criminal prosecution giving an embryo special status outside the

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human body. Other key principles included patients and donors to be offered counseling and for them to provide informed consent prior to treatment or donation. There was a recommendation for the need to consider the welfare of any child to be born as a result of fertility treatment. One of the main recommendations was that a statutory licensing authority should be established to regulate both research and those treatment services which were recommended to be subject to control.

It was recognized that legislation based on the recommendations would take time to formulate, so in 1985 the Voluntary Licensing Authority was established as an interim measure (known latterly as the Interim Licensing Authority). This was a joint initiative by the Medical Research Council and the Royal College of Obstetricians and Gynecologists. The committee, consisting of both clinical and lay members, provided a mechanism to inspect centers and provide advice [2].

Founding of the HFEA and the 1990 Act

Following extensive consultation by the UK government in 1986 and the publication of a government white paper in 1987, the Human Fertilization and Embryology Bill received Royal Assent in 1990. The 1990 Act was therefore the first legislative framework to govern assisted reproduction and associated research in the UK. The Act permitted certain activities to be carried out under a license and prohibited certain other activities.

The 1990 Act also provided for the establishment of the Human Fertilization and Embryology Authority (HFEA), an executive, nondepartmental public body founded in 1991. This was the first regulatory body of its kind in the world. The HFEA's remit under the legislation of the 1990 Act regulates clinics by licensing:

- 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 made up of a chair and members. The 22 members are appointed through the Appointments Commission and are from a variety of backgrounds including scientific, clinical, legal and lay. The board meets to authorize authority papers and also offer advice through the several committees. The members remit is to set the strategic direction of the Authority, be responsible for audit and governance, and to make licensing decisions.

Licensing UK Centers

In order to carry out assisted reproduction techniques in the UK, centers must be licensed by HFEA (HFE Act 1990 amended). Once a license application is made by a center, the HFEA will carry out an inspection to assess compliance against the Act and requirements of the Code of Practice. Once the inspection is complete, a report is produced, and the licensing decision is finally made by either the executive licensing panel of the HFEA or a license committee made up of members. Following the initial grant of a license, there is a requirement for a center to have an inspection at least every 2 years. At each inspection the center has to demonstrate compliance. The current compliance cycle for issuing licenses which commenced on the 1st of April 2010 is a 4-year cycle. Centers are asked to complete an online self-assessment questionnaire (SAQ) to assess their own compliance against regulatory requirements. The SAQ is fed into a risk tool combined with general performance indicators to determine the inspection focus. At the beginning of the 4-year cycle, an initial inspection is carried out focusing on the core requirements from the Act, any important themes and areas of concern. After 2 years, the SAQ is repeated, and an interim inspection is conducted to focus on themed areas set by the HFEA and any areas for concern. At anytime within the 4-year cycle, a targeted inspection can occur if an incident occurs or an investigation is required. Inspections can also occur unannounced [3]. Following amendments made to the 1990 Act by 2007 Regulations, any center providing any services that assist a woman to carry a child (nonmedical fertility services) are now required to have a license.

Changes in Legislation

Multiple Birth Rates

Restriction on the number of embryos transferred after IVF in order to reduce the number of multiple pregnancies has formed part of the regulatory program since the Warnock report. The VLA produced the first guidelines regarding the number of embryos to transfer in 1987, with transfer of a maximum of three embryos unless exceptional circumstances were evident. Improvement in the overall success rate of IVF led to the number of embryos to transfer being reduced to 2 in the 1990s.

In 2008 the "one at a time" initiative has firmly placed the concept of single embryo transfer as a primary principle for centers (www.oneatatime.org.uk). The HFEA founded an expert group to advise on the implications of single embryo transfer. Following the report [4], a stakeholder group (http://www.oneatatime.org.uk/145.htm) was formed of representatives from the different professional bodies to provide advice on strategies for single embryo transfer and to support the initiative. The HFEA is committed to the policy and issued directions requesting all centers to produce a minimization of multiple births policy by January 2009 [5]. The Authority has set yearly targets for multiple birth rates, with the aim to reach a target of a multiple birth rate of less than 10%.

Donor Anonymity

Another major change of direction in legislation has been the issue of donor anonymity. The Warnock report recommended that it was good practice for donor to be unknown to the couple throughout treatment, and that remaining anonymous was appropriate as it offered protection from both legal and emotional complications. This was further recommended by the VLA whose guidelines stated donation should be anonymous and known donation avoided. However, based on the principle of the child's right to know its origins, regulations allowing the details about egg donors and sperm donors to be passed on to the offspring, including the name and last address of the donor came into force after the 1st of April 2005, removing the concept of donor anonymity [6].

2008 Act

Since the 1990 act became law there have been major developments in science, clinical practice, and public attitudes and therefore in January 2004 the government announced a review of the 1990 Act. After public consultation and a review of the 1990 Act by the House of Commons Science and Technology Committee, the Government published the white paper [7]. Following this the draft Bill was published in 2007. After scrutiny, the government introduced a new bill to reform the legislation. The Bill received Royal Assent in November 2008, and the new provisions came into force on the 1st of October 2009.

The main changes to the 2008 Act include:

- A change to the licensing procedure
- To ensure that the creation and use of all human embryos outside the body regardless of how they are created is subject to regulation
- A ban on selecting the sex of offspring for nonmedical reasons
- Retention of the duty to take into account of the welfare of the child when providing fertility treatment, but the removal of the reference to the "need for a father" and replacement with the concept of "supportive parenting"
- Provisions to recognize same sex couples as legal parents of children conceived through the use of donated sperm, eggs, or embryos
- Altering restrictions on the use of HFEA collected data to make it easier to do follow-up research, for example, on the long-term effects of IVF
- Provisions clarifying the scope of legitimate embryo research activities, including explicit rules on embryo testing
- Allowing embryos to be used for training purposes
- Changes to the statutory storage limits
- Code of Practice

Section 25 of the 1990 Act requires the HFEA to maintain a Code of Practice to help clinics comply with regulatory requirements to enable them to carry out licensable activities. The first edition published in 1991 was part of a manual to help centers understand the licensing process. The eighth edition of the Code [8] was published on the 1st October 2009 and comprises regulatory requirements and guidance notes. The code is comprehensive and encompasses all clinic activities including:

- Staffing
- Counseling
- Information and consent
- Multiple births
- Welfare of the child
- Embryo testing
- Donation and surrogacy
- Use of gametes and embryos
- Research and training
- Facilities and administration
- Treating people fairly
- Records and other obligations

The Code of Practice has specific guidance: examples are listed below.

Storage of Gametes and Embryos

The center has to have documented procedures to ensure that the processes involved in gametes or embryos being placed into storage comply with the regulatory requirements, and that procedures to ensure relevant consent are in place. The facility and equipment has to be suitable and validated, and all storage vessels have to be fitted with alarms. All patients have to be screened prior to storage for HIV1 and 2, Hepatitis B and C, and in high-risk patients, HTLV1.

Amendments to the Human Fertilization and Embryology Act 1990, which came into force on 1 October 2009, changed the statutory storage period for embryos from 5 to 10 years. The new Human Fertilization and Embryology Regulations [9] also made it possible to extend storage of gametes and embryos for a maximum of 55 years. Gametes and embryos can therefore be stored for a maximum of 55 years provided that at each 10-year check point the center 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. This now allows "mother to daughter" donation of cryopreserved oocytes, for example, where the daughter has Turner syndrome and will therefore not be able to produce oocytes herself.

Provision of Information and Consent

The law is very specific that all individuals seeking treatment which will involve the creation of embryos, or any form of gamete or embryo donation must be provided with appropriate information. Written informed consent must also be gained before treatment commences and gametes are procured. In taking consent it is expected that patients are provided with sufficient information so they are able to understand the nature, purpose and implications of the proposed treatment or donation. They should also have had the opportunity for counseling and understand that they can vary or withdraw consent.

Witnessing

The implications of inadvertent mixing of gametes of embryos resulting in the birth of a child with the "wrong" genetic parents are obvious. In order to minimize this risk, it is a mandatory requirement that centers have witnessing systems to double check both the identity of patients and their samples [8]. The protocols employed must cover all steps in the treatment pathway which are listed in full in the Code of Practice [8], and fully traceable records must be made by the person performing the procedure and the person carrying out the witnessing procedure. Some of the witnessing steps may be replaced by electronic witnessing systems but full risk assessments must be carried out prior to these being integrated into clinical practice.

Information and Data Submission

The legislation states that centers are legally obliged to submit certain data to the HFEA. This includes not only the information the HFEA is required to hold on its register but also information about adverse incidents and near misses. With regard to treatment information, there is an electronic data interchange system for the submission of specific forms relating to the different stages of a treatment cycle within defined time scales.

European Directives

In 2007, IVF centers in the UK had to comply with the introduction of Directive 2004/23/EC and the commissioning directives 2006/17/EC [10] and 2006/86/EC [11]. Although across Europe, the actual implementation date was a year earlier, as UK centers had the HFEA regulatory framework centers were allowed a year's delay to prepare. These directives amended the HFE Act 1990 and laid down standards of quality and safety and the technical requirements for human tissues and cells intended for human application. The HFEA as the competent authority were nominated to ensure compliance against the directive.

The purpose of the directive was to establish a uniform standard across Europe and involved specifying the grade of laboratory air quality with respect to air quality, both for nonviable and viable particle counts.

Other Accreditation Schemes

Many clinics seek to optimize their quality management in order to provide the safest and most efficient service to their patients. A number of different accreditation and certification schemes exist to ensure high-quality service provision. Many IVF centers in the UK chose to be ISO9001:2008 certified. This enables the unit to meet the requirements of the license condition to have a quality management system and also provides both patients and purchasers with assurance that the system is robust and of a high standard.

The Association of Clinical Embryologists and British Fertility Society have a joint policy and practice committee to produce good practice guidelines. Although not legislatory, these papers offer advice regarding best clinical practice. The professional bodies also provide comprehensive training and validation programs to ensure that the requirements of the Code of Practice are met. In the UK, embryologists must become state registered within 1 year of becoming eligible. The shortest route to registration is to complete the ACE certificate training program, and after a period of 4 years, state registration can be attained by passing a viva examination with assessors from the Association of Clinical Scientists. Once passed, a recommendation is sent to the Health Professions Council to award state registration.

Conclusion

The HFEA currently provides a comprehensive regulatory mechanism which ensures patient protection. Mechanisms are available either to place conditions on center's license or withdraw a license if noncompliance against the legislation is demonstrated. The regulatory framework ensures high standard of practice and ensures research is ethically acceptable within our society. Although seen by some as over bureaucratic, the system has been widely emulated in other countries and reassures the public that this sensitive and ethically challenging area of medical practice is performed to the highest standards.

Acknowledgments The future of the HFEA is currently uncertain as the HFEA was identified as one of the public bodies to be placed in the Public Bodies Act 2011 which gives Ministers the power to abolish,

merge, modify the public bodies and their offices. It also means that functions of Public Bodies can be modified or transferred and consequential provisions can be made. Within Schedule 5 the HFEA is listed as one of the PB's that ministers can modify or transfer the functions and offices.

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Regulation, Licensing, and Accreditation of the ART Laboratory in Europe

69

Julius Hreinsson and Peter Sjoblom

Abstract

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 between the European countries. The ESHRE Web site gives an excellent illustration of the situation in the various countries in Europe in this respect. 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, etc. Therefore, we have decided to focus here on common requirements with reference to the EU Tissues and Cells Directive and give further details in a summary at the end of each section of the chapter. Although some laboratories in Europe may currently be operating without fulfilling these standards in all details, they will in the coming years be expected to do so.

Keywords

EU assistive reproductive technologies regulations • Directives • Requirements • European ART laboratory regulations • ISO accreditation • Tissue directive in ART • Personnel in the European ART laboratory

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 between the European countries. The ESHRE Web site gives an excellent illustration of the situation in the various countries in Europe in this respect [1].

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, etc. Therefore, we have decided to focus here on common requirements with reference to the EU Tissues and Cells Directive [2] and give further details in a summary at the end of each section of the chapter. Although some laboratories in Europe may currently be operating without fulfilling these standards in all details, 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, e.g., Magli et al. [3].

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 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.

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Acknowledging that the present chapter perhaps includes some of the less accessible and yet very important pieces of information in the book, we have decided to try and increase ease of reading by starting each subchapter with a description of the subject matter and include details in tables and examples.

The Tissue Directive and ART

The tissue directive was conceived to provide a regulatory framework for the foreseen rapid development of regenerative medicine (http://europa.eu/rapid/pressReleasesAction.do?reference=IP/04/85&format=HTML&aged=0&language=EN;&guiLanguage=en). 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 has consulted with experts in the respective areas and the technical directives, which guide the implementation of the tissue 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 tissue directive for implementation in assisted reproduction.

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 does often 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, 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 recently established certification program will form 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.

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 some guidance can be 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. 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 then 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.

Facilities

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 air flow required to maintain such conditions [4]. The final version of the EU directive which, with annexes [2, 5, 6], is in force allows however 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 implies 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 levels of 3,520,000 particles larger than 0.5 μm, 29,000 particles larger than 5 μm, and 100 colony-forming units 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 a minimum of 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—i.e., 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, alarm 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 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 69.1, the standards for laboratory quality for the ART clinic and laboratory are specified.

ART Facilities

ART clinics must have designated and suitable facilities for their operations.

Rooms for oocyte pick up and embryo transfer, laboratory facilities for culture, processing, storage, cryopreservation, etc., must fulfill at least class D (ISO class 8) air quality,

Table 69.1 Standards of laboratory quality for the ART clinic and laboratory

	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

although local higher standards may be applied. In practice, this is in most cases taken to mean:

- Restricted access
- Changing room must be close to the laboratory/operation room
- HEPA-filtrated ventilation
- Positive pressure from lab/operation to surroundings
- Conditions must be specified with regards to the above points as well as:
  - Particle counts and microbial count
  - Rules for access must be documented
  - Hygienic standards must be established
  - LAF hoods must be used for cell culture work

Equipment and Materials

One purpose of the tissue directive is to minimize the risk of adverse effects on the tissue by handling, processing, or storage. 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 14 June 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 its 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 has 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.

Traceability

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 traceable. When the tissue is collected, moved, used, or discarded, this must be recorded 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 would ensure 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, e.g., 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. Particular regulations apply for the export and import of tissues to and from other countries.

Documentation

As mentioned above, the tissue establishment must have a documented system for quality management. This system should contain all guiding documents, i.e., instruction how to perform a procedure. The system should also contain all reporting documents, i.e., forms. All documents should be reviewed regularly and reauthorized. This is to ensure that the documents are up to date, 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, e.g., 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 patients 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.

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 (SOPs, standard operating procedures). Computerized solutions may be of great assistance in this respect. Other aspects

Table 69.2 Comparison of quality requirements of the EU directive and the ISO 9001 standard

	Directive 2004/23/EC	ISO 9001
Document all procedures and SOPs	X	X
Document control	X	X
Document check lists	X	X
Training manuals	X	X
Guidelines	X	X
Nonconformities	X	X
Internal/external audits	X	X
Process analysis		X

of quality control are, e.g., registering and process of nonconformities, internal and external revisions, and continuous improvements in processes and procedures. In Table 69.2, the requirements of the EU directive in this respect are specified and simplified as a comparison to the ISO 9001 standard.

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 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.

Coding

A single European coding system is to be established for all tissue establishments. Currently, this is not in use for couples attending treatment using their own gametes (partner donation); however, this may be implemented in years to come. The coding system will be obligatory for sperm donation and oocyte donation. This is not implemented as yet so further specifications regarding practical details will have to await further developments in this area. Which coding system will be used has yet to be decided; international standards such as ISBT 128 have been discussed since there are practical advantages with implementing a system which already is in use in other areas of laboratory medicine. 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-code 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.

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 [7].

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.

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, 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 is currently determined by individual countries, but at the time of writing, the EU has raised the prospect of testing being made mandatory before each treatment cycle.

Donors need to be screened prior to donation, the donated tissues then need to be quarantined for 6 months and the donor re-tested 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.

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.

Legal Aspects

Although implementation of the EU directive on tissues and cells calls for coordination of regulatory demands on ART in Europe, local legislation may vary considerably. This is especially clear regarding the use of donated gametes and whether donor anonymity is allowed. Time limits for cryostorage of oocytes and embryos vary considerably; possibilities for applying embryo selection are in some cases restricted, and the use of preimplantation genetic diagnosis is limited in some countries. Embryo research is another area where legislation may impose restrictions. Instead of attempting to describe this constantly changing European legal landscape in detail, we refer to the ESHRE Web site (www.eshre.com) where members can obtain detailed and updated information regarding the legal situation in various European countries.

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Regulation, Licensing, and Accreditation of ART Laboratories in India

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B.N. Chakravarty and Rita Modi

Abstract

In India, until recently, the ethics related to the practice of ART have been governed by customary social practices within the community, conventional human rights, and sometimes religious law, which are based on spiritual values. The extent of human rights is acceptable in Indian law provided the procedures of investigations and their consequence do not harm anybody or contradict the customary practices and do not exceed the limits of popular tolerance. Social and customary practices in India are dominated mostly by religious instincts. It is the purpose of this chapter to review how different legislations as well as socioeconomic, demographic, and religious factors influence the access to and the practice of ART with special emphasis in Indian context.

Keywords

Indian ethical ART guidelines • Socioeconomic, social, religious factors • Regulation of ART in India • ART laboratory regulation and licensing • Licensing of ART labs in India

In India, until recently, the ethics related to the practice of ART have been governed by customary social practices within the community, conventional human rights, and sometimes religious law, which are based on spiritual values. The extent of human rights is acceptable in Indian law provided the procedures of investigations and their consequence do not harm anybody or contradict the customary practices and do not exceed the limits of popular tolerance. Social and customary practices in India are dominated mostly by religious instincts. It is the purpose of this chapter to review how different legislations as well as socioeconomic, demographic, and religious factors influence the access to and the practice of ART with special emphasis in Indian context.

Though currently not existing, a new legislation is about to be implemented for the ethical practice of ART in India. Indian Council of Medical Research (ICMR) in collaboration with National Academy Medical Science (NAMS) had appointed an expert group committee consisting of 19 members in 2002 with a view of formulating the national guidelines on "Accreditation, Supervision and Regulation of Infertility/ART Clinics in India." Through several meetings held in Mumbai and Delhi, the members of the expert group committee have compiled a draft which has been cleared through Human Rights Commission and scrutinized by another expert group committee. It has been presented in the parliament and is awaiting final approval.

The members of the expert group committee have very carefully scrutinized the current practice of IUI and ART in India. There are about 150 or may be even more ART and infertility clinics all over the country. Some of these clinics are well equipped and render services to the patient in the way in which it is expected to be done. Other clinics are still in the initial phases of their experience and expertise, and they are yet to reach the normal standard of professional perfection of this subspecialty. This has been the reason for drawing up uniform guidelines for ethical practice of acceptable IUI/ART methods in the country. India is a vast country with wide ranges of variable social customs and religious instincts. Obviously for such major step, the document/draft needs to

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be widely debated and discussed by as many interested persons concerned as possible.

Therefore, the contents of the draft have been extensively debated in different metropolitan cities of the country namely Kolkata, Bangalore, Mumbai, Delhi, Jodhpur, Chennai, and Hyderabad. Though, unanimity could not be reached on certain controversial issues, yet it has been realized and accepted by the majority of the participants in the public debates that such a guideline is urgently needed for the ethical practice of IUI and ART in our country.

General Considerations

Importance and Significance of Guidelines

The desire to have children by infertile couple is so desperate that many clinics with little expertise or reliability are forced or tempted to treat patients in the way in which ideally or ethically they should not be treated. Guidelines may help both patients and clinics to avail the facilities of *Tier* system approach for the stepwise management of infertile couples.

There are certain gray areas in the *substitutional* management of infertility such as the use of donor's sperm, donor's eggs, surrogacy, etc. Guidelines have laid down certain codes of ethics which will maintain a uniform policy with due regard to their long-term consequences. These guidelines will help both the clinician and the patient.

A stepwise approach will help the clinicians for better management of infertile couples. The patients will also be confident to avail the facilities of rational treatment, which the couple demands and deserves at a particular level of infertility care unit. Guideline has elaborated "three-tier" system of infertility management. Depending on the severity of the cause of infertility, patient can attend the specific "level" of infertility care unit, or she may be referred to the appropriate infertility clinic by the family physician to whom they first go for primary consultation.

Categorization of Infertile Couples

Broadly speaking, infertile couples may be categorized into three groups:

- Those having a single defect either in the male or in the female partner. This single defect may also be either easily treatable or untreatable. Examples of single treatable defect are ovulatory dysfunction, mild oligospermia, or minor anatomical defect like tough hymen in the wife or phimosis in the husband. Single untreatable defect includes azoospermia, premature ovarian failure, or absence of uterus and vagina.
- Infertile couples with multiple defects. Examples of such defects include azoospermia in husband and tubal block in wife. They will ordinarily not respond to either medical or surgical treatment. ART or adoption will be the rational choice.
- The third group of infertile couples are those who do not clinically exhibit any apparent abnormality and still they are unable to conceive. They are grouped under the nomenclature of unexplained infertility.

The different categories of infertile couples with broad outline of management protocol are summarized in Fig. 70.1.

Classification of Infertility Clinics

Depending on the severity of the cause of infertility, the patients may be treated by their gynecologist or physician to whom they may go first, and when necessary, they may be referred to infertility care unit where appropriate facilities for investigation and treatment for that patient would be available. Accordingly, infertility care units have been categorized into four levels and have been authorized to offer treatment as applicable to each of these levels. The different levels of infertility care units have been designated as level IA, level IB, level II, and level III.

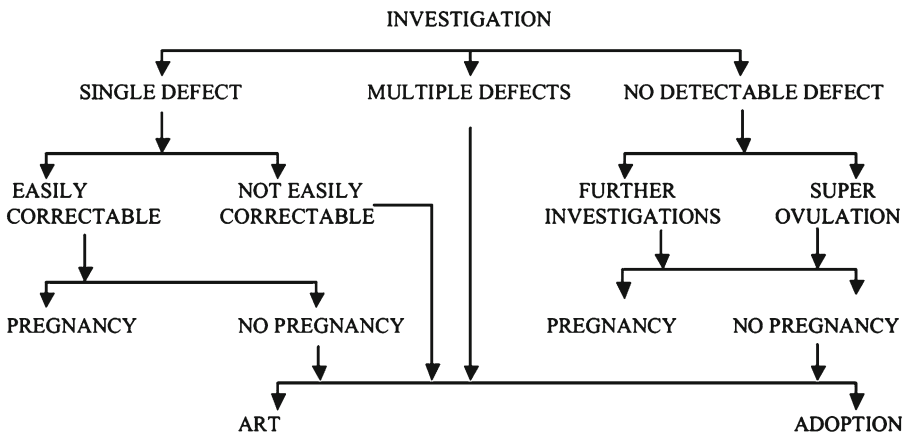

Fig. 70.1 Categories of infertile couples and broad outline of management protocol

The gynecologists or the physician in charge of level IA infertility care unit should have an appropriate postgraduate degree or diploma with adequate knowledge of reproductive endocrinology and imaging. Level IA infertility care units will not be involved in sperm manipulation for the purpose of IUI and will not require an accreditation under the guideline. Level IB infertility care units are entitled to perform IUI under the supervision of a gynecologist with a postgraduate degree. Level IB infertility care unit (like those of level II and level III) will need accreditation. More difficult cases like apparently untreatable single defect in either partner or multiple defects should be referred to higher levels of infertility care units (level 1B, level II, and level III) and those requiring extra corporeal gamete manipulation.

Guidelines for IUI

Accreditation

In the various public debates, held in different parts of the country, there were bitter controversies over the issue on IUI. IUI involves male gamete manipulation and laboratory processing of the sperms. In the initial drafts, the procedure of IUI was not included in the category of level I infertility care unit. It was debated that a gynecologist working at level I infertility care unit should be allowed to perform IUI and no regulatory supervision by the accreditation committee should be imposed on such treatment (IUI). Following these debates, a compromising guideline has been proposed and formulated.

> For AIH, it has been proposed that a gynecologist with adequate experience in reproductive endocrinology and imaging is competent enough to perform IUI; he or she does not require accreditation. But the laboratory where the semen is being processed and prepared needs accreditation.

For AID, accreditation of the center, (level IB) laboratory, and sperm bank is essential, and international norms as outlined below are required to be strictly maintained.

Sperm Donor

Use of sperm donated by a relative or a known friend of either the wife or the husband shall not be permitted. It will be the responsibility of the ART clinic to obtain sperm from appropriate banks; neither the clinic nor the couple shall have the right to know the donor identity and address, but both the clinic and the couple, however, shall have the right to have the fullest possible information from the semen bank of the donor such as age, height, weight, skin color, blood group (ABO and Rh), educational qualification, profession, family background, freedom from any known disease or carrier status, medical or genetic (such as hepatitis B or AIDS) diseases, ethnic origin, and the DNA fingerprint (if possible), before accepting the donor semen. Age of the donor should not be below 21 and above 45 years. It will be the responsibility of the semen bank and the clinic to ensure that the couple does not come to know the identity of the donor. The ART clinic will be authorized to appropriately charge the couple for the semen provided and the tests done on the semen donor.

Requirements for Semen Bank

- Either an ART clinic or a law firm or any other suitable independent organization, like an NGO (nongovernmental organization), may set up a semen bank. If set up by an ART clinic, it must operate as a separate entity.
- The bank will ensure that suitable record of all donors is kept for 10 years, after which, or if the bank is wound up during this period, the records should be transferred to an ICMR repository.
- A bank may advertise suitably for semen donors who may be appropriately compensated financially.
- On request for semen by an ART clinic, the bank will provide the clinic with a list of donors (without the name or the address but with a code number) giving all relevant details. The semen bank shall not supply semen of one donor for more than ten successful pregnancies. The bank shall keep a record of all semen received, stored, and supplied and details of the use of the semen of each donor. This record will be liable to be reviewed by the accreditation authority.
- The bank must be run professionally and must have facilities for cryopreservation of semen, following internationally accepted protocols. Each bank will prepare its own SOP (standard operation procedures) for cryopreservation.
- Semen samples must be cyropreserved for at least 6 months before first use, at which time the semen donor must be tested for HIV and hepatitis B and C.
- The bank must ensure confidentiality in regard to the identity of the semen donor.
- A semen bank may store preparation for exclusive use on the donor's wife. This is applicable for couples, where husband is professionally busy and may not be present at the time of wife's insemination (IUI, IVF, or ICSI). An appropriate charge may be levied by the bank for the storage. In the case of nonpayment of the charges when the donor is alive, the bank would have the right to destroy the semen sample or give it to a bonafide organization to be used only for research purposes. In case of death of a donor, the semen would become the property of the legal heir or nominee of the donor nominated at the time the donor gives the sample for storage to the bank. All other conditions that apply to the donor would now apply to the

legal heir, excepting that he cannot use it for having a woman of his choice inseminated by it.
- All semen banks will require accreditation.

Guidelines for ART: Criteria for Accreditation and Licensing

There are many areas in the proposed guideline, which have been thoroughly discussed and elaborately debated either at in-house expert committee meetings or at the public debates. The optimum acceptable views on controversial issues have been documented with due regard to their long-term legal, ethical, and social consequences. Few of them are mentioned below.

Physical Requirements for an ART Clinic

A well-designed ART clinic of level II or level III should have a nonsterile and a strictly sterile area as detailed below. Some of the spaces mentioned below could be combined (i.e., the same space may be used for more than one purpose) as long as such a step does not compromise the quality of service. However, the space provision for the sterile area cannot be combined with those for the nonsterile area and vice versa.

The Nonsterile Area Must Include the Following Things

- A reception and waiting room for patients.
- A room with privacy: Adequate measures must be taken to ensure that history taking and examination are carried out in strict privacy, maintaining the dignity of the patients. In case of a male doctor examining a female patient, there must be a female attendant present. The room must be equipped with an examination table and gynecological instruments for examining the female patient. It is advisable to have an ultrasonography machine with a probe (transvaginal in case of female partner evaluation) for better assessment of reproductive organs of male and female partners. A color Doppler would be useful but not essential.
- A general-purpose clinical laboratory.
- Store room: Facility must be available for storing sterile item (media, needles, catheter, Petri dishes, etc.) and nonsterile material under refrigerated and nonrefrigerated conditions as appropriate.
- Record room: Record keeping must be computerized as far as possible so that data are accessible retrospectively for analysis. There are many software programs for this purpose, which are commercially available today. A user-friendly one should be chosen that could be used widely. Besides containing essential details of the patient's records, it must contain history of the cause of infertility as diagnosed earlier, results of new diagnostic procedures if relevant, the treatment option best suited for the particular patient, the treatment carried out and the outcome of treatment, and follow-up if any. Any other noteworthy point, such as possible adverse reaction to drugs, must be recorded. ICMR is making an effort to devise a form for basic data recording, which would be suitable for India.
- Autoclave room: A separate facility must be available for sterilizing and autoclaving all surgical items as well as some of those to be used in the in vitro culture laboratory.
- Steps for vermin proofing: Adequate steps should be taken to make the whole clinic vermin proof, with suitable traps for preventing insects and other forms of unwanted creatures entering the clinic. This essential detail should be planned at an early stage because no pesticide can be used in a fully functional IVF clinic, as it could be toxic to the gametes and embryos.
- Semen collection room: This must be a clean room with privacy and an appropriate environment; it should be located in a secluded area close to the laboratory. Such a facility must be available in-house rather than having the patient collect the sample and bring it to the laboratory for analysis, as in the latter case, semen quality is likely to be compromised. Procedures for collection of semen as described in the WHO Semen Analysis Manual must be followed with special reference to the type of container used; the containers must be sterile, maintained at body temperature, and nontoxic. The room must have a wash-basin with availability of soap and clean towels. The room must also have a toilet and must not be used for any other purpose.
- Semen processing laboratory: There must be a separate room with a laminar airflow for semen processing, preferably close to the semen collection room. This laboratory must also have facilities for microscopic examination of postcoital test smears. Good Laboratory Practice (GLP) guidelines as defined internationally must be followed. Care must be taken for the safe disposal of biological waste and other materials (syringes, glass slides, etc.). Laboratory workers should be immunized against hepatitis B and tetanus.
- Clean room for IUI: There must be a separate area/room with an appropriate table for intrauterine insemination (IUI).

The Sterile Area

The sterile area shall house the operation theater, a room for intrauterine transfer of sperm or embryos, and an adjoining embryology laboratory. Entry to the sterile area must be strictly controlled by an anteroom for changing footwear, area for changing into sterile garments, and a scrub station. The sterile area must be air-conditioned where fresh air

filtered through an approved and appropriate filter system is circulated at an ambient temperature of 22–25°C.

- The operation theater: This must be well equipped with facilities for carrying out surgical endoscopy and transvaginal ovum pick-up. The operation theater must be equipped for emergency resuscitative procedures.
- Room for intrauterine transfer of embryo: This room must be a sterile area having an examination table on which the patient can be placed for carrying out the procedure and rest undisturbed for a period of time.
- The embryology laboratory complex: The embryology laboratory must have facilities for the control of temperature and humidity and must have filtered air with an appropriate number of air exchanges per hour. Walls and floors must be composed of materials that can be easily washed and disinfected; use of carpeting must be strictly avoided.

The Embryology Laboratory Must Have the Following

- A laminar flow bench with a thermostatically controlled heating plate
- A stereomicroscope
- A routine high-powered binocular light microscope
- A "high-resolution" inverted microscope with phase contrast or Hoffman optics, preferably with facilities for video recording
- A micromanipulator (if ICSI is done)
- A CO_2 incubator, preferably with a back up
- A hot air oven
- A laboratory centrifuge
- Equipment for freezing embryos in a programmed manner
- Liquid nitrogen can
- A refrigerator

Appropriate steps need to be taken for the correct identification of gametes and embryos to avoid mix-ups. All material from the operation room, culture dishes and Falcon tubes for sperm collection (including lids), must bear the name of the patient. In the incubator, identified oocytes and sperm should be kept together on the same tray and double-checked. Pipettes used should be disposed off immediately after use. The embryology laboratory must have a daily logbook in which all the day's activities are recorded, including the performance of the equipment.

Ancillary Laboratory Facilities

The infertility clinic need not have in-house facilities to perform all the procedures necessary to diagnose infertility, such as those mentioned below. They can be farmed out to specialty laboratories specializing in delivering such services, as long as they are located in the neighborhood.

- Hormone and other assays: The infertility clinic must have ready access to laboratories that are able to carry out immunoassays of hormones (FSH, LH, prolactin, hCG, TSH, insulin, estradiol, progesterone, testosterone, and DHEA) and tests such as for HIV and hepatitis B. Endocrine evaluation constitutes an essential diagnostic procedure to determine the cause of infertility. It is also necessary to estimate blood estradiol in samples taken from a woman undergoing controlled ovarian hyperstimulation, and have the result on the same day to determine the dose of drugs to be given for induction of ovulation. Accurate monitoring of endocrine response to controlled ovarian stimulation goes a long way in preventing ovarian hyperstimulation.
- Microbiology and histopathology: Another important facility in an ART clinic (or easily accessible to it) would be that of a microbiology laboratory that can carry out rapid tests for any infection and a clinical chemistry laboratory. Facilities for carrying out histopathological studies on specimens obtained from the operation theater would also be desirable.
- Maintenance of the laboratories: Each laboratory should maintain in writing standard operating manuals for the different procedures carried out in the laboratory. It should be ensured that there is no "mix up" of gametes or embryos. The patient's name should be clearly labeled on all the tubes, dishes, and pipettes containing the gametes and embryos. All pipettes should be immediately discarded after use. Laminar flow hoods, laboratory tables, incubators, and other areas where sterility is required must be periodically checked for microbial contamination using standard techniques, and a record of such checks must be kept. A logbook should be maintained which records the temperature, carbon dioxide content, and humidity of the incubators and the manometer readings of the laminar air flow. All instruments must be calibrated periodically (at least once every year) and a record of such calibration maintained.
- Quality of consumables used in the laboratory: All disposable plasticware must be procured from reliable sources after ensuring that they are not toxic to the embryo. Culture media used for processing gametes or growing embryos in vitro should be preferably procured from reliable manufacturers. Each batch of culture medium needs to be tested for sterility, endotoxins, osmolality, and pH. The embryologist should know the composition of the media that are being used. Most media are supplemented with serum; they should, therefore, be tested for antibodies to HIV 1 and 2, hepatitis B surface antigen, and hepatitis C RNA.

Backup Power Supply

There should be no interruption in power supply to the incubator and to other essential services in the clinic. Given the power supply situation in India, it is, therefore, imperative that a power backup in the form of UPS systems and/or a

captive power generation system is available in infertility clinics offering ART services.

Personnel: Essential Qualifications of ART Team

The practice of ART requires a well-orchestrated teamwork between the *gynecologist*, *the andrologist, and the clinical embryologist* supported by a *counselor* and a *program coordinator/director*. The staff requirements given below would be mandatory for level II and level III clinics. In the case of small level II and level III clinics, the services of the andrologist, the clinical embryologist, and/or the counselor could be shared.

Gynecologist

The minimal qualification for a gynecologist in a level IB, level II, or level III clinic is a postgraduate diploma or degree in gynecology. Additional experience should include:

- Understanding the causative factors of male and female infertility.
- Acquiring knowledge of the practice and use of diagnostic methods for determining the cause of infertility.
- Acquiring knowledge of the clinical aspects of reproductive endocrinology and the reproductive defects caused by endocrine factors, and an understanding of the limitations of the currently used hormone assay methods and of the techniques available for medically or surgically correcting endocrine disorders.
- Acquiring competence/skills in gynecological ultrasonography to diagnose reproductive tract anomalies, monitoring ovarian and uterine response to ovarian stimulation, picking up oocytes at the most appropriate time, and transferring embryos by any one of the several methods currently available to handle embryo transfer in "difficult cases."
- The gynecologist must be well versed, particularly in the pharmacology of hormone action, and know how to avoid situations such as ovarian hyperstimulation syndrome that can pose a great health hazard.

The responsibilities of the gynecologist would include the following:

- Interviewing of the infertile couple initially
- History taking
- Physical examination of the female
- Recommending appropriate tests to be carried out, interpreting them, and treating medical disorders (infections, endocrine anomalies)
- Carrying out laparoscopy or sonohysterosalpingography for determining the status of the uterus and the fallopian tube
- Advising the couple on planned relationship in simple cases
- Carrying out AIH, AID, IUI, IVF, or ICSI as the case may warrant, based on diagnostic evidence

In case of male factor infertility, if the gynecologist is confident and competent, he/she can treat such cases or refer them to the andrologist. The treating doctor must be responsible for maintaining all records of diagnosis, treatment given, and consent forms. Before any treatment is given, it is advisable that the couple is referred to the counselor, with all the details of the case, for proper advice and counseling. It would be the gynecologist's responsibility to see that all equipment and instruments in the operation theater are properly functional and in order and that a logbook is maintained of their use and operation.

Andrologist

Fifty percent of infertility cases are related to male factors, many of which can be treated by specific ART procedures or other less invasive procedures. Andrology, a subject related to male reproduction, does not constitute a formal course in the medical curriculum in India, although several journals in Andrology are published from different parts of the world including China. There is also an International Andrological Society with branches or affiliated societies all over the world. In India, it is the urologist with a postgraduate degree in urology that often takes on the task of treating male infertility. Such individuals must receive additional training in diagnosis of various types of male infertility covering psychogenic impotence, anatomical anomalies of the penis which disable normal intercourse, endocrine factors that cause poor semen characteristics and/or impotence, infections, and causes of erectile dysfunction.

- The andrologist must have knowledge of the occupational hazards, infections, and fever that cause reversible or irreversible forms of infertility and knowledge of ultrasonographic or vasographic studies of the reproductive excurrent ducts to detect partial occlusion that can be surgically corrected.
- He/she must understand the principles of semen analysis and their value and limitation in diagnosis of male fertility status. The person should also be able to interpret the fertility status of the male from the result of semen analysis. The andrologist must be able to collect semen by prostatic massage for microbial culture in cases where infection may lie in the upper regions (prostate, seminal vesicles) of the reproductive tract. He/she should also be able to collect spermatozoa from the excurrent ducts or testis for use in ICSI and must also be knowledgeable about the genetic implications of using poor-quality sperm for ICSI as this technique can vertically transfer the genetic defects of the father to the child. He/she should be familiar with the surgical procedures available for correcting an anatomical defect in the reproductive system such as epididymovasal reanastomosis and varicocoelectomy.
- An individual may act as an andrologist for more than one clinic, but each clinic where the andrologist works must

own responsibility for the andrologist and ensure that the andrologist is able to take care of the entire workload of the clinic without compromising on the quality of service. The responsibilities of the andrologist would include the following:

- Recording case histories
- Prescribing appropriate diagnosis and treatment based on the diagnosis
- Carrying out such surgical procedures as warranted by the diagnosis
- Maintaining all the records, from the case history to the treatment given, and the patient consent forms
- Referring the couple to the gynecologist for carrying out the appropriate ART procedure, if necessary, after the male factor has been duly investigated
- Referring the couple to the counselor if necessary
- In cases of surgical intervention, making sure that the operation theater is fully functional and all supplies are available before the start of any surgical procedure
- Entering any deficiency that needs attention in the operation theater logbook

Clinical Embryologist

The clinical embryologist must be knowledgeable in mammalian embryology, reproductive endocrinology, genetics, molecular biology, biochemistry, microbiology, and in vitro culture techniques. The biologist must also be familiar with ART. He/she must be either a medical graduate or have a postgraduate degree or a doctorate in an appropriate area of life sciences. (In the case of a clinic in existence for at least 1 year before the promulgation of these guidelines, a person with a B Sc or BV Sc degree but with at least 5 years of first-hand, hands-on experience of the techniques mentioned below and of discharging the responsibilities listed below would be acceptable for functioning as a clinical embryologist in the particular clinic. Such persons would also be eligible to take a test to be designed and conducted by an appropriate designated authority to qualify for a position of a clinical embryologist in a new clinic.) He/she must be familiar with the following:

- Principles and practice of semen analysis and cryopreservation of semen
- Cytology of mammalian and human oocyte to identify stages of oocyte maturation accurately
- All aspects of embryology including developmental biology
- Cell biological techniques used in cell and tissue culture
- Molecular biology and genetics of human reproduction
- Micromanipulation of sperm and oocytes for carrying out ICSI and single-cell biopsies of embryos for preimplantation genetic diagnosis
- Principles and functioning of all the equipment used in the laboratory
- In vitro fertilization of oocytes after processing the gametes
- Principles and practice of embryo freezing

The responsibilities of the clinical embryologist would be:

- To ensure that all the necessary equipments are present in the laboratory and are functional.
- To perform all the procedures pertaining to processing, handling, and culturing of gametes and embryos in the laboratory and hand over the embryo to the gynecologist.
- To maintain records of all the procedures carried out in the laboratory.
- In case of shortage of adequately trained clinical embryologists, an individual may act as a clinical embryologist for more than one clinic, but each clinic where the person works must own responsibility for the embryologist and ensure that the embryologist is able to take care of the entire workload of the clinic without compromising on the quality of service. An embryologist must not be associated with more than two centers at any given time.

Counselors

Counselors are an important adjunct to any infertility clinic. Indeed, in the UK, counselors are appointed by the clinic, but they report to an independent body. This ensures that there is fair play by the clinic, and the patients are adequately informed of what and what not to expect from the treatment offered to them. Counseling for ART is not taught as a separate subject anywhere. A person who has at least a degree (preferably a postgraduate degree) in Social Sciences, Psychology, Life Sciences, or Medicine and a good knowledge of the various causes of infertility and its social and gender implications, and the possibilities offered by the various treatment modalities, should be considered as qualified to occupy this position. The person should have a working knowledge of the psychological stress that would be experienced by potential patients, and should be able to counsel them to assuage their fears and anxiety and not to have unreasonable expectations from ART. A member of the staff of an ART clinic who is not engaged in any other full-time activity in the clinic can act as a counselor. The counselor must invariably appraise the couple of the advantages of adoption as against resorting to ART involving a donor. An individual may act as a counselor for more than one clinic, but each clinic where the counselor works must own responsibility for the counselor and ensure that the counselor is able to take care of the entire counseling load of the clinic without compromising on the quality of the counseling service.

Program Coordinator/Director

This should be a senior person who has had considerable experience in all aspects of ART. The program coordinator/director should be able to coordinate the activities of the rest of the team and take care of staff administrative matters, stock keeping, finance, maintenance of patient records,

statutory requirements, and public relations. He/she should ensure that the staff is keeping up with the latest developments in their subject, by providing them with information from the literature, making available to them access to the latest journals, and encouraging them to participate in conferences and meetings and present their data. The program coordinator/director should have a postgraduate degree in an appropriate medical or biological science. In addition, he/she must have a reasonable experience of ART.

Audit

For renewal of accreditation and licensing, periodic monitoring of quality control and quality assurance is absolutely essential.

Quality Control in ART Laboratory

This is the process by which all aspects of the program are monitored and confirmed that they are functioning within acceptable limits. The quality control should assure that the program is operating in a stable and reproducible fashion. Without this stability in the program, it becomes very difficult to determine whether an unusual outcome for a particular patient is due to a patient-related issue or due to a programmatic failure.

Quality Assurance in ART Laboratory

The objective of quality assurance is not only to maintain the standard (quality control) but also to improve the outcome. From that point of view, quality control is a part of quality assurance. Therefore quality assurance includes quality control assessment of personnel, procedures, and materials, which provide data for improvement of activities.

Guidelines/Regulations on Controversial Issues in ART

Oocyte Donation or Embryo Donation

Oocyte donation (OD) would necessitate using the husband's semen for fertilization and transferring the resultant embryo to the infertile female partner. Embryo donation (ED) would obviate the necessity of using the husband's semen. The choice of oocytes and embryos for oocyte or embryo donation would depend entirely on the circumstances prevalent at the time the infertile couple comes for treatment and the access of the infertility clinic to frozen oocytes or embryos.

Indications for oocyte or embryo donation:

- Gonadal dysgenesis
- Premature ovarian failure
- Iatrogenic (due to ovarian surgery or radiation, or chemical castration) ovarian failure
- Women who have resistant ovary syndrome or who are poor responders to ovulation induction
- Women who are carriers of recessive autosomal disorders
- Women who have attained menopause

Donors should be healthy (as determined by medical and psychological examination, screening for STDs, and absence of HIV antibodies) women in the age group of 18–35 years. She should be married having at least one child. Oocytes are commonly obtained from a willing donor on "hired" basis. As oocyte cryopreservation is not popular in India, maintaining the anonymity of oocyte donor may not always be possible. The recipient should be a healthy woman (determined by medical and psychological examination) having normal genitalia (as determined by physical examination) and uterine cavity (as determined by hysterosalpingography).

In case of OD, the semen characteristics of the husband must be determined to see if they are in conformity with those associated with normal fertility. The blood group of the donor should be noted; the donor should also be tested for antibodies to rubella, HIV, hepatitis, CMV, gonorrhea, syphilis, chlamydia, mycoplasma, and trichomonas. Ovum/embryo donation can be carried out in menopausal women with no surviving child and desiring to have a child. The endometrium of menopausal women has the ability to respond to sex hormones and provide a receptive environment for the implantation of an embryo. Various protocols are now available to prepare the endometrium of the recipient for OD or ED with estrogens and progestogens until the placenta takes over the function of maintaining the gestation.

Oocyte Sharing

The system of oocyte sharing in which an indigent infertile couple that needs to raise resources for ART agrees to donate oocytes to an affluent infertile couple wherein the wife can carry a pregnancy through but cannot produce her own oocyte for in vitro fertilization with the sperm of the male partner of the affluent couple, for a monetary compensation that would take care of the expenses of an ART procedure on the indigent couple, must be encouraged.

Surrogacy: Sourcing of Donor Oocytes and Surrogate Mothers

Law firms and semen banks will be encouraged to obtain (e.g., through appropriate advertisement) and maintain information on oocyte donors and surrogate mothers. The above organizations may appropriately charge the couple for providing an oocyte donor or a surrogate mother. The oocyte

donor may be compensated suitably (e.g., financially) by the law firm or semen bank when the oocyte is donated. However, financial negotiation between a couple and the surrogate mother must be conducted independently between them.

The surrogate mother may be known to the commissioning couple—either a friend or relative, or may have been unknown to them prior to surrogacy arrangement. There are conflicting views about this. Some believe that arrangement with unknown surrogate mother may create problem because the ultimate outcome of handing over the baby to the genetic parents will depend on trust between strangers. In other forms of assisted reproduction, involving gamete donation, the donor generally remains anonymous. But in surrogacy, a "forced friendship" must be established between a previously unknown surrogate mother and the commissioning couple. On the other hand, problems may also arise when surrogate host is selected from commissioning couple's own family or friends. Sometimes this may complicate normal life within the family often to a damaging extent. In Israel, law has been made in such a way that the commissioning couple will not like a relative to become a surrogate mother because the law in this country accepts surrogate mother as the real mother.

In India, regarding sourcing of surrogate host, ICMR guidelines suggest the following:

- Surrogate mother will be procured by law firms or semen banks. All semen banks or law firms require accreditation.
- However, negotiation between a couple and the surrogate mother must be conducted independently between them.
- Payments to surrogate mothers should cover all genuine expenses associated with pregnancy. Documentary evidences of financial arrangement for surrogacy must be available. The ART center should not be involved in this monetary aspect.
- Advertisement regarding surrogacy should not be made by the ART clinic. The responsibility of finding a surrogate mother, through advertisement or otherwise, should rest with the couple or a semen bank.
- The bank will ensure that all criteria (age screening for medical/genetic disorders, HIV, hepatitis B, hepatitis C, etc.) are met and suitable records of surrogate mother are kept for 10 years, after which, or if the bank is wound up during this period, the records should be transferred to the ICMR repository.
- The bank may advertise suitably for surrogate host who may be appropriately compensated financially.
- A surrogate mother should not be over 45 years of age. Before accepting a woman as a possible surrogate for a particular couple's child, the ART clinic must ensure (and put on record) that the woman satisfies all treatable criteria to go through a successful full-term pregnancy.
- In Indian context, a known person, as well as a person unknown to the couple, may act as a surrogate mother for the concerned infertile couple. In the case of a relative acting as a surrogate mother, the relative should belong to the same generation as the woman desiring the surrogate.
- A prospective surrogate mother must be tested for HIV and shown to be seronegative for the virus just before embryo transfer. She must also provide a written certificate that (a) she did not have a drug intravenously administered into her through a shared syringe, (b) she has not undergone blood transfusion, and (c) she or her husband (to the best of his/her knowledge) has had no extramarital relationship in the last 6 months (this is to ensure that the person would not come up with symptoms of HIV infection during the period of surrogacy). The prospective surrogate mother will also declare that she will not use drugs intravenously and not undergo blood transfusion excepting of blood obtained through a certified blood bank.
- No woman may act as a surrogate more than thrice in her lifetime.

From monetary point of view, Indian regulation at the moment is unlike those existing in UK, and we have more or less followed USA principle of "hired" surrogacy.

A surrogate mother carrying a child biologically unrelated to her must register as a patient in her own name. While registering, she must mention that she is a surrogate mother and provide all necessary information about the genetic parents such as names, addresses, etc. She must not use/register in the name of the person for whom she is carrying the child, as this would pose legal issues, particularly in the untoward event of maternal death (in whose names will the hospital certify this death?). The birth certificate should be in the name of the genetic parents. The clinic, however, must also provide a certificate to the genetic parents giving the name and address of the surrogate mother. All the expenses of the surrogate mother during the period of pregnancy and postnatal care relating to pregnancy should be borne by the couple seeking surrogacy. The surrogate mother would also be entitled to a monetary compensation from the couple for agreeing to act as a surrogate; the exact value of this compensation should be decided by discussion between the couple and the proposed surrogate mother. An oocyte donor must not act as a surrogate mother.

A third-party donor and a surrogate mother must relinquish in writing all parental rights concerning the offspring and vice versa. Indian guideline has suggested that "A child born through surrogacy must be adopted by genetic (biological) parents unless they can establish through genetic (DNA) fingerprinting (of which records will be maintained by the clinic) that the child is theirs."

This issue becomes somewhat complicated when commissioning couples desire a pregnancy not only through surrogacy but with oocyte donation as well. It is desirable though not always possible or practical to procure eggs for an anonymous donor. The anonymous donor is arranged by

the accredited semen bank or a law firm. The donor's eggs are then fertilized with the husband's sperm of the commissioning couple. The resulting embryo(s) are then transferred into a surrogate host, who is also arranged by the accredited semen bank. This means that "surrogate host" and "oocyte donor" are separate individuals helping an infertile woman who has neither a uterus nor any oocyte.

Ideally and legally, babies born through such type of surrogacy need to be adopted. Commissioning couples will not like adoption. The commissioning couple has accepted this complicated procedure only because the procedure will be more socially acceptable than the previously practiced "natural" or "partial" surrogacy. In this desperate situation, in order to avoid future legal complications, the following procedures may be suggested:

- The surrogacy arrangement between the commissioning couple and surrogate host should be made in the presence of a lawyer.
- Donor if procured by the semen bank should sign the agreement of relinquishing her right on the resulting offspring in presence of a lawyer. If this is done, question of adoption does not arise. Legally the commissioning parents are not bound to disclose the name of the oocyte donor. But medical records, social status, and other information regarding the donor which will be available in the same bank (organization which has procured the donor) may be communicated to the child if occasion arises when the child becomes major (after the age of 18 years).

Experience and information about psychological aspects of surrogacy are very limited. The merits and demerits of following aspects of gestational surrogacy may require further evaluation.

Maintaining Contact with Surrogate Mother After the Child Is Born

While some people report benefits achieved by maintaining contact between the parties, others feel that this does not suit everybody.

Disclosure to the Family Members and to the Child About Surrogacy Arrangement

This is also controversial. Studies of families where a child has been created by gamete donation indicate that majority of parents do not wish to disclose the method of conception to the child, though recently, there has been a greater tendency for open discussion with family members and the child. It has been suggested that secrecy about the conception method may have a negative impact on the child's psychological development. Evidence from research on adoptive families indicates that children are more likely to develop emotional and behavioral problems when their parents conceal about the adoptions. Surrogacy resembles adoption; therefore, it may be argued that children are likely to fare better when the fact about surrogacy is disclosed to them at a very early age. The surrogate mother feels better when she discloses her surrogacy agreement to other members of the family.

Right of a Child Born Through Various ART Technologies

A child born through ART shall be presumed to be the legitimate child for the couple, having been born in wedlock and with the consent of both the spouses. Therefore, the child shall have a legal right to parental support, inheritance, and all other privileges of a child born to a couple through sexual intercourse. Children born through the use of donor gametes and their "adoptive" parents shall have a right to available medical or genetic information about the genetic parents that may be relevant to the child's health.

Children born with the use of donor gametes shall not have any right whatsoever to the identity (such as name, address, parentage, etc.) of their genetic parent(s). A child thus born will, however, be provided all other information about the donor as and when desired by the child, when the child becomes an adult. While the couple will not be obliged to provide the above "other" information to the child on their own, no deliberate attempt will be made by the couple or others concerned to hide this information from the child as and when asked for by the child. In case of a divorce during the gestation period, if the offspring is of a donor program—be it sperm or ova—the law of the land as pertaining to a normal conception would apply.

ART in Unmarried Women

All along, society has been used to the concept of childbirth occurring within the bonds of bisexual wedlock. Gays and lesbians in the West have assumed the right to marry, and some gays have even gone to the extent of seeking out a woman who would donate her oocyte for in vitro fertilization and a surrogate mother who would carry the transferred IVF embryo pregnancy to term on behalf of gay couple. Similarly, lesbians and women preferring to be single parents have sought and succeeded in getting ART clinic to artificially inseminate them to bear a child. Gays, lesbians, and single mothers are gaining ground to get social approval of their sexual preferences around the world. The days are not far off when they too would make demands on ART clinics in countries such as ours as their Western counterparts have done.

AIH or ART in HIV-Positive Women

This is not permissible in our country as per decision of the Supreme Court in the case of X vs. Hospital 2 (1998) and section 269.

Embryo Reduction and Sex Selection

Due to high incidence of multiple gestations in ART, embryo reduction has an important place, but long-term follow-up of children from continuing sacs remains long overdue. In India, a human is considered to exist when the fetal heart starts beating, that is, about 30 days after fertilization, as per prevailing religious decree. Hence, despite being socially unacceptable, it is allowed in principle and by law due to benefit to the continuing fetuses and welfare of the mother. For gender selection, female feticide was practiced in parts of India. This has been legally banned except for medical indications, for example, sex-linked medical disorders.

Embryo Research

The ICMR is considering the following statutory guidelines relating to embryo research and embryo donation:

- There should be a statutory regulation whereby embryology laboratories shall have complete freedom to treat surplus embryos with the consent of donors either for research purposes or for donation to a suitable recipient without any commercial involvement in these procedures.
- The regulatory climate will improve if a code of secrecy and freedom of use of embryo and prohibition of commercial use is introduced.

Single Embryo Transfer

Elective single embryo transfer is practiced in countries like Sweden, Belgium, and few other European countries, but in India, there is no specific law regarding this. Nevertheless, by prevailing customs, the number of embryos transferred has been limited to three or four. The purpose is to avoid multiple pregnancies with its associated risks and complications, including those associated with the procedure of fetal reduction.

Ethical and Moral Responsibilities of ART Clinics

The moral and ethical responsibilities and the legal rights of the third parties in ART, such as semen donors, oocyte and embryo donors, and the surrogate mothers, are already a cause of much concern to our present society. Technical developments are occurring quite rapidly in ART, offering the hope of parenthood to an increasing number of infertile couples. An example would be the enabling of menopausal woman to become pregnant. More recently, the issues of therapeutic and reproductive cloning, which now is possible, have raised new moral, ethical, social, and legal conflicts. The question is how does an ART clinic respond as a part of society to such situations. Medically, there is no great technology involved. How would our society treat babies born out of wedlock? One must remember that India is a country where the cult of "Hijras" or eunuchs has been socially accepted for centuries. Can our country also absorb such types of parents? In such a situation, it is considered wise not to make any one of the possibilities as illegal but step up appropriate counseling facilities to ensure that there is least suffering on the part of those directly or indirectly involved.

Institutional Ethics Committees

Each ART clinic of levels IB, II, and III must have its own ethics committee constituted according to ICMR guidelines, comprising reputed ART practitioners; scientists, who are knowledgeable in developmental biology or in clinical embryology; a social scientist; a member of the judiciary; and a person who is well-versed in the comparative theology. Should the local ART clinic have difficulty in establishing such a body, the accreditation authority should constitute such a body, co-opting a representative of the ART clinic.

Possible Misuse of ART: Sale of Embryos and Stem Cell

The stand taken by the foreign governments on embryo research opens up the possibilities of embryos from developing countries that do not have appropriate national guidelines in this area, are being commercially exploited, and sold to foreign countries. Therefore, sale or transfer of human embryos or any part thereof, or of gametes in any form and in any way—that is, directly or indirectly—to any party outside the country must be prohibited. Within the country, such embryos or gametes could be available to the bonafide researchers only as a gift, with both parties (the donor and the recipient) having no commercial transaction, interest, or intent.

Providing ART Services to the Economically Weaker Section of the Society

Offering Services at Cost Price or Exploring the Scope of Low Cost of IVF

Setting up of a modern ART clinic and running it satisfactorily is an expensive affair, requiring dedicated staff that would render long-term services. The setting up of ART clinics in public sector, which does not exist as of now, must be explored. Meanwhile, the government could also explore the possibility of requesting the already established ART

clinics that are willing to serve the weaker sections at cost price that would be met by the government. With the introduction of Gn-Rh antagonists, it is expected that new stimulation protocol may be rescheduled or explored using clomiphene/letrozole in combination with few ampoules of gonadotropin and at the same time maintaining an acceptable pregnancy rate.

Reduction of Drug Cost

The concerned ministries must take a look at the reason for the high cost of ovarian stimulation hormones and encourage and support local pharmaceutical industries to start manufacturing human menopausal gonadotropin. Pure/rec follicle-stimulating hormone should be manufactured indigenously so that the treatment of our infertility patients is not dictated by the commercial motives of the multinational companies but by national needs.

Establishing a National Database for Human Infertility

Unfortunately, there is no documented database available in our country that would cover data on all aspects of infertility, and there is an urgent need for the same. It is worrisome to see that, with the primary aim of providing a child to the infertile couple, a variety of sophisticated ART procedures are being used to overcome male factor infertility without understanding the underlying cellular and molecular etiology. In the process of curing infertility in the patient, there is a high iatrogenic risk of transmitting an abnormal parental genotype to the ART-born child. An appropriate database would allow the quantification of such risks.

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Troubleshooting in the Clinical Embryology Laboratory: The Art of Problem-Solving in ART

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Kathryn J. Go, Jay C. Patel, and Rick Dietz

Abstract

An underachievement in positive clinical outcomes in assisted reproductive technology (ART) is intolerable and demands an investigation into what factor(s) may be causing or underlying it. A well-written quality control program provides not only the requirements of an optimally functioning ART laboratory but also the infrastructure for querying the performance of each element, whether technical operator, instrument, supply, or material in the effort to identify, isolate, and provide an effective remedy to it. The potential advantage of considering causes outside the laboratory is also advanced in consideration of the multidisciplinary and multifactorial nature of ART. The use of evidence, i.e., data, elucidating outcomes stratified by both technical/laboratory and medical operators, as well as by an array of patient demographic and clinical factors, may enhance not only the occasional need to troubleshoot in an interval of concern but to keep the ART clinic most knowledgeable about all of the facets of its practice. In driving a troubleshooting effort, leadership, collegiality, and a methodical plan are all important in its success.

Keywords

Troubleshooting in the ART laboratory • Quality control in the ART laboratory • Personnel in the ART laboratory • Materials and equipment in the ART laboratory • Instrumentation in the ART laboratory

A laboratory for assisted reproductive technology (ART) that may be underperforming, or perceived to be underperforming, causes a disconsolation that is uniquely difficult to bear! As the central operating system in providing infertility therapy through in vitro fertilization (IVF), the ART laboratory will usually immediately come under scrutiny with the hypothesis that some technical part is failing or faulty. To quell the turbulence and return it to optimal performance, a method by which the laboratory's individual components can be assessed to identify or rule out a potential problem is invaluable in answering the questions, "What is wrong with the lab? Is the factor global or specific, chronic or acute?"

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When the need arises to shoot and neutralize trouble in the clinical embryology laboratory, the best arm to take up is the laboratory's quality control (QC) program. In its specific provisions and requirements for the laboratory to operate optimally, the QC program is a ready list by which to methodically check each element in the context of the whole.

Quality control (QC) is a discipline-specific program describing what an organization must have and do to consistently deliver a service or product of highest quality. In ART, a field uniting medicine and laboratory science in patient care, the requirements to achieve QC are both broad and deep and have been recognized from the inception of this discipline and continuously addressed [1–10]. In this chapter, the authors will describe what should be included in a QC program for the ART laboratory, and how this can provide the framework and checklist for troubleshooting. A methodology to systematically address all the elements

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of the laboratory's and clinic's operation can be catalytic in this endeavor when the most rapid identification and effective remedy of a problem is sought. Both contemporary and early literature references are provided to reflect the history of establishing QC for IVF laboratories.

The Dual Nature of the ART Laboratory

A unique feature of ART is that it fulfills two distinct but related objectives: It is both diagnostic and therapeutic. In its role of acquiring, examining, "processing," and uniting human sperm and eggs under defined culture conditions, the ART laboratory can identify and substantively influence the attainment of critical benchmarks in the reproductive process: maturation, fertilization, and the course of embryo development—a diagnostic function. In the culture of human embryos, the ART laboratory can attribute some potential for implantation and pregnancy and uses that information for embryo selection for transfer to the patient—a therapeutic function.

During its evolution of the last 30-plus years, the ART laboratory has expanded its repertoire of techniques and obtained an array of equipment, supplies, materials, and expertise required for advanced methodologies. In its earliest days, IVF, the insemination of eggs with sperm, comprised the only form of ART: the placement of egg-containing cumulus masses in a culture dish with tens of thousands of motile sperm from the husband, partner, or sperm donor to allow fertilization to occur [11–13]. A significant technical and proficiency distance has been traversed from this point with the advent of embryo cryopreservation and the various methodologies requiring expertise in cell micromanipulation: assisted hatching of the zona pellucida, intracytoplasmic sperm injection (ICSI), and embryo biopsy for preimplantation diagnosis or screening of the embryo. The ART laboratory is also continually challenged to appraise and adopt techniques that will enhance or supplant existing methods, such as in vitro maturation of eggs and egg and blastocyst vitrification. This ever-increasing complexity of ART demands that the QC program evolve in parallel to be effective.

The Components of an ART Laboratory and Ensuring Their Quality

An ART laboratory's function is realized through these five general components: (1) physical plant and its support; (2) instrumentation, supplies, and materials; (3) qualified and specifically trained personnel; (4) a laboratory mission statement and manual; and (5) optimal integration of the laboratory into the general clinical model for delivery of patient care through common objectives, communication, and documentation.

Clinical embryologists are fortunate in having multiple resources to consult, including a well-developed literature on the subject in the form of medical and scientific articles, manuals and textbooks [14–18], guidelines developed by professional societies [19–22], and atlases [23, 24]. These provide requirements and recommendations governing these general areas as well as benchside references.

Physical Plant and Its Support

The installation of the ART laboratory must be underpinned by a design plan providing:

1. Adequate space: An ART laboratory entails multiple large instruments that must be installed and mounted securely and in a way that allows optimal operation, access, and easy maintenance. Of equal importance is the provision of space for the embryologists to move about the laboratory and use the instruments without constriction of movement.
   - Are instruments appropriately arranged?
   - Is workflow optimized by laboratory space and layout?
2. Appropriate proximity to operating/clinical procedure rooms: Given the sensitivity of human gametes and embryos to changes in temperature, the maximally expedient transport of them from point of collection or placement to and from the laboratory, respectively, is required [25].
   - Are distances between retrieval and embryo transfer room to laboratory appropriate?
   - Are distances between incubator and microscope or laminar flow hood appropriate?
3. Ventilation and optimal air quality and climate stability: To protect the egg, sperm, and embryo cultures from fluctuations in temperature and potential noxious substances in ambient air (e.g., fumes from organic solvents in paints or cleaning solutions or even personal cosmetics, or motor vehicle exhaust from proximity to a building entry or parking area), the ART laboratory should have its own air handling systems augmented with filtration, humidification, and individual heating and cooling controls [26–28].
   - Is air quality in the laboratory protected from contamination by organic solvents, noxious cleaning materials, vapors, dust, etc.?
4. Appropriate light: Illumination in the laboratory should be adjusted to a level that allows examination of all materials by the embryologists with the avoidance of intensity thought to be deleterious to reproductive cells or tissue [29, 30].
   - Are ambient light levels in the laboratory appropriate?

5. Adequate electrical capacity with a source of emergency power: The design of the ART laboratory should take into account the number of instruments and their individual electrical power demands and have an appropriate number and placement of electrical outlets, and the installation of an emergency power generator.
   - Can electricity be provided to critical instruments during a power outage?
   - Can transient loss of power or out-of-range condition to an instrument be detected?
6. Plumbing capacity: Appropriate sinks and faucets are essential in any laboratory and are also integral to the laboratory's requirement for an eye-washing station and potential desire for water processing by deionization or filtration [31].
   - Is the water processing system functioning properly?
7. Monitoring and alarm systems: To provide protection and preservation of the patient samples, a system by which critical instruments and storage facilities are constantly monitored for adherence to a desired set point or range is required. The alarm situation can summon most rapid attention to resolve the out-of-parameter condition.
   - What is the nature and duration of any out-of-range condition for an instrument?
   - What effect could be exerted by an out-of-range condition of the instrument?
8. Support through an extramural department of engineering or maintenance: The partnership of a facility's engineers or maintenance staff is vital in assisting the laboratory staff in seeking immediate repairs or temporary solutions to deviations in the laboratory's physical plant, e.g., loss of heat, water, or power or physical integrity such as problems in wall or floor structure.

Instrumentation, Supplies, and Materials

Early practitioners of clinical embryology will recall that the first IVF laboratories were often located in converted storage spaces or allotted the most minimal of space allowing for one or two incubators, a laminar flow hood, dissecting and inverted microscopes, a centrifuge, a refrigerator, a programmable cell freezer and liquid nitrogen source, a cryopreservation storage tank, some shelving and a modicum of bench space.

In contrast, the anticipated inventory of a twenty-first century, full-service ART laboratory includes an extensive inventory of instruments and adjunct resources. All instruments, specifically those that are electronically programmed to maintain conditions within a tight range of parameters, must be monitored to ensure adherence to those conditions. Among these are culture incubators, refrigerators, and cell freezers.

Following the calibration of the incubator or freezer to the desired parameters, e.g., $\%CO_2$, chamber temperature and relative humidity of the incubator, and internal temperature of the refrigerator, a daily log of these measurements and their verification must be maintained. Handheld infrared readers have largely replaced Fyrite solution measurements for carbon dioxide percentage in incubator air, and certified thermometers in each chamber provide temperature readings. For those laboratories with adequate funding, continuous electronic monitoring and recording of all instruments can be achieved through both hardwired and wireless systems.

Programmable cell freezers can be linked to printers for monitoring and validation of each run. Regularly scheduled diagnostic evaluations and calibration by authorized service technicians for each instrument are required to ensure optimal operation and prevent failure from mechanical or electronic wear and fatigue.

When indicated, replacement of aging instruments or instruments approaching obsolescence is an excellent strategy for avoiding their breakdown and deriving the benefits of most current models and technological advances.

Complete and detailed records on the life history of each instrument must be maintained and archived after the instrument is retired or discarded as part of global laboratory records.

When troubleshooting, evaluation of each instrument can be made by a checklist such as this:

1. Incubators:
   - Are internal chamber surfaces clean?
   - Are incubators calibrated to and maintaining appropriate settings?
   - Are gas and temperature levels confirmed by independent means, e.g., chamber thermometer and carbon dioxide infrared detector, respectively?
   - Is humidification source (water reservoirs) clean and free of microorganisms or particulate matter?
   - For triple-gas incubators, are gases provided in correct proportions?
2. Inverted microscopes with a system for maximal optical resolution such as a Hoffman modulation system and camera/monitor/visual image recording:
   - Is the optics system correctly set for optimal resolution?
   - Is the stage warmer temperature appropriately set and maintained?
   - Is the light intensity appropriately set?
3. Dissecting microscopes:
   - Is the stage warmer temperature appropriately set and maintained?
   - Is the light intensity appropriately set?
4. Micromanipulators:
   - Are the controls functioning properly and providing best operation?
   - Are the instruments, e.g., holding, ICSI, hatching, biopsy pipettes, appropriately crafted with correct diameters, bevels, angles, and polishing?

5. Laminar flow hoods:
   - Is velocity of airflow correctly set?
   - Are any heated surfaces appropriately calibrated for temperature setting and maintenance?
   - Is the surface temperature independently verified by a thermometer?
6. Refrigerators:
   - Are the temperatures of both refrigerator and freezer compartments set appropriately and verified using thermometers?
7. Mobile microscope workstations enclosed in a heated chamber such as an Isolette:
   - Is the stage warmer temperature appropriately set and maintained?
   - Is the ambient temperature within the chamber appropriately set and maintained?
8. Centrifuges:
   - Are the rotor speeds used for sperm preparation appropriately set?
   - Is the ambient temperature of the centrifuge chamber maintained within an acceptable range during the centrifugation intervals for sperm preparation?
9. Cell freezers:
   - Are the individual ramps of the freezing program appropriate and correctly entered into the instrument?
   - Have the temperatures in the freezing temperature been verified by a thermocouple?
   - Is each freezing fun run recorded and verified for correct progress?
10. Storage tanks for cryopreserved samples:
    - Are liquid nitrogen levels or vapors adequately maintained?
    - Are cryopreserved samples appropriately placed and immersed in the tanks?
11. LASER for zonal opening:
    - Are the settings for the LASER correct for strength and pulse duration?
    - Is the LASER calibrated for target precision?
12. Instrument for visualization of meiotic spindle:
    - Is location of meiotic spindle attained for purpose of sperm injection?

Materials and Supplies in the ART Laboratory

The array of ART-specific culture media, disposable culture ware, and supplies and accessories has steadily increased and become available to clinical embryologists through both specialized and general vendors of laboratory supplies.

There may still be a small number of ART laboratories that prefer to prepare and test their own culture, cryopreservation, or micromanipulation (polyvinylpyrrolidone [PVP], acid Tyrode's for hatching or biopsy) media, but those that purchase these through industrial sources may find more convenience through time- and effort-savings. Industrial manufacturers and distributors of ART culture and cryopreservation media perform and document the toxicity testing that is vital to an ART laboratory's confidence in using these products. These Certificates of Assurance reflect testing through appropriate bioassays, most frequently a mouse one-cell or two-cell embryo assay, and usually govern large lots of a specific component. Tracking of lot numbers and dates of application affords the ability to identify potentially underperforming products and their withdrawal, if warranted, from clinical use.

The following are useful to list and track for start and end dates and clinical outcomes obtained with each, if desired. A troubleshooting effort demands a scrutiny of these components given their integral role in gamete and embryo management:

- Culture media
- Any holding media for oocytes or sperm, such as HEPES-buffered media
- Media used during oocyte retrieval or as a base solution for cryopreservation media, e.g., phosphate buffered saline (PBS)
- Sperm preparation media for sperm washing or isolation, such as products for filtration through density gradients
- Cryopreservation solutions used for sperm, egg, or embryo freezing
- Solutions used for ICSI such as hyaluronidase and polyvinylpyrrolidone (PVP)
- Oil used for culture drop overlay
- Biopsy medium

Some laboratories will augment this testing by independent, repeat testing under their own auspices, possibly using an alternate bioassay such as sperm motility that may detect a potential toxin or contaminant [32–34].

For any materials for which testing by vendor or manufacturer is not available, the laboratory's independent survey and quarantine of these until the bioassay threshold is attained or surpassed is good practice to survey their inventory of disposables. The consideration of all contact materials should be made during any troubleshooting survey and may include but is not limited to:

- Pasteur pipettes, pipette tips, or any instruments/supplies used to handle and transfer gametes and embryos
- Serological pipettes
- Graduated cylinders
- Culture dishes or plates for egg and embryos
- Culture tubes
- Culture flasks
- Micromanipulation instruments such as holding, hatching, biopsy, and ICSI pipettes or the glass from which they are fabricated
- Straws of vials for gamete or embryo cryopreservation
- Protective globes used by the embryologist during procedures

Personnel

While there are a few specific formal programs for clinical embryology in the United States, a reasonable assessment is that the training of these vital personnel is still achieved by on-site "apprenticeship" education in existing laboratories. This phenomenon is the result of the history of IVF in the United States that reflects the recruitment of clinical embryologists from the ranks of reproductive biologists, animal science laboratory specialists, basic reproductive science research technicians, and medical technologists and technicians. Indeed, the first clinical embryologist and laboratory director of an embryology laboratory in the United States was Lucinda Veeck, MLT (ASCP), Ph.D. (hon.).

Each laboratory will develop its own philosophy and mission of excellence, and this will be reflected in the quality of its technical training and commitment to its quality control and improvement programs.

The foundation of the training program is the laboratory manual, a compendium of all its protocols and procedures describing (a) the name of the technique; (b) its purpose; (c) what materials are required to perform it; (d) a step-by-step description of the technique from start through completion; (e) a method by which its completion can be qualitatively and quantitatively assessed; (f) a checklist by which progress in training can be monitored and evaluated; and (g) a list of resources for the operator to consult in the event of a problem encountered during the procedure ensuring its appropriate and acceptable completion.

A laboratory manual for ART may be expected to include some or all of these techniques:

- Preparation of culture media
- Oocyte retrieval from ovarian follicular aspirates
- Isolation of sperm from semen, epididymal fluid, or testicular biopsies
- Insemination of eggs
- Intracytoplasmic sperm injection
- Assessment of eggs for fertilization or alternate post-insemination states such as polyspermy
- Evaluation and selection of embryos or blastocysts for intrauterine transfer or cryopreservation
- Cryopreservation or vitrification
- Gamete or embryo thawing
- Embryo or blastocyst biopsy for preimplantation testing
- Preparation of embryo biopsies for preimplantation testing
- Maintenance of the laboratory and its instruments
- Record-keeping
- Communication within the laboratory and with clinical partners (physicians and nurses)

When suboptimal clinical outcomes are encountered, technical performance will usually immediately come under scrutiny. The concern is that during the course of in vitro culture or manipulation, some negative event is befalling the gametes or embryos. While an array of styles, approaches, and emphases may characterize the same procedure, the following principles and goals are universally applied and sought, respectively, in technical execution, and each should be specifically investigated during troubleshooting:

- Adherence to the protocol provided in the laboratory manual.
- Minimization of exposure of the gametes or embryos to ambient conditions.
- Optimal manipulation of gametes and embryos: gentle, rapid transfers from culture plate to culture plate, or transfer catheter.
- Optimal manipulation of eggs and sperm during ICSI or of embryos during hatching, biopsy.
- Proper preparation for performance of a technique, e.g., alignment of instruments, application of laser for hatching or biopsy.
- Optimal environment in laboratory conditions for operator and instrument performance.
- Results in a program by which technical performance is constantly peer-surveyed for proper execution and the opportunity for improvement, and periodic review of those records.
- Results in an approved program of proficiency testing for embryo culture and review and documentation of acceptable results with corrective action when required.
- Consideration of appropriate staffing for volume of laboratory work to avoid operator fatigue as in other technically intense fields [35].

Laboratory Records and Documentation

Integral to a program of QC are complete and meticulous records; the following are requisite to best laboratory practice and will assist in troubleshooting:

1. Patient records: life histories of all eggs and accounting for all samples
2. List of instruments and their maintenance records
3. Inventory of all laboratory materials and supplies, certificates of assurance, lot numbers, usage dates, recall notices
4. Log of laboratory incidents, events, or milestones to capture the daily experience of a laboratory and evaluate for trends
5. Personnel records including educational credentials, certifications, continued education, training, and continued proficiency reviews
6. Daily instrument checks
7. File of all current forms used for documentation and records

8. Chain of command and identification of personnel and responsibilities/approval to perform laboratory techniques
9. Licenses, registrations, and certificates of registration
10. Laboratory meeting minutes and documentation of completion of tasks and objectives outlined there

Troubleshooting: A Multiperspective Approach

Suboptimal performance in an ART center reflected in decreased clinical parameters such as fertilization or implantation rates may come not only from a laboratory-based source (e.g., an environmental or technical factor), but possibly from a clinical or practice-based factor. A holistic approach toward the evaluation and monitoring of the variables contributing to an ART center's outcomes fosters an integrated, team approach toward quality controls and improvement. Active participation by reproductive endocrinologists, clinical and laboratory staff, and the chief executive of an ART center is vital to the effectiveness of such an approach. In conjunction with a thorough examination of laboratory factors, the consideration of patient demographics, approaches to controlled ovarian hyperstimulation, numbers of embryos transferred, and clinical procedures (such as embryo transfer) may allow an ART center to have the most complete understanding of its operation and factors affecting outcomes.

A highly effective and efficient construct for such a multidimensional, interdisciplinary approach is the continuous quality improvement initiative. Building upon a center's existing QC program, to include new perspectives and a broader examination of center's policies and practices, allows for a deeper understanding of factors contributing to both success and failures. Such an expansion of the QC program should include two discrete areas of focus: (1) longitudinal trends and (2) acute events.

The examination and reporting of longitudinal trends raises the visibility and ownership of quality across the organization, making quality control and improvement a primary strategic goal for the ART center. The consistent presentation of data, allowing for fact-based analysis and discussion, is a vital tool in a center's pursuit of improving quality. Such data should be readily shared across departments, unblinded and otherwise transparent, but presented within the construct of quality improvement in order that individuals, clinical teams, and departments recognize the proactive, strategic purpose of such data sharing. While difficult to introduce at first, with resistance from some clinicians certain to be encountered, an organization's willingness to openly and honestly assess performance, with center leadership taking an active and ongoing role in analyzing performance trends, greatly increases the probability of measureable performance improvement over time.

On occasion, failures will occur within a center, leading to risk management episodes and challenging staff performance review. How such acute events are managed, however, can significantly impact the outcome of the event as well as avoidance of similar future failures. The incorporation of event reporting and review into a center's QC program also significantly increases the probability of error reduction and improved performance. Each event should be followed by a thorough investigation, preferably yielding a root cause analysis of the event. Such a rigorous approach allows for vital teaching moments with staff, while also potentially uncovering process failures and opportunities to introduce or reinforce standardization. Corrective actions should be implemented, including staff retraining or changes in processes. While each staff member must ultimately own their performance failures and be able to demonstrate personal performance improvement, a constructive, performance improvement approach, rather than a blame-game approach, reinforces a culture committed to quality improvement and a willingness to engage in open, honest assessment of workflow, protocols, and clinical performance. The organization's response and corrective actions should also be openly reported through the center's QC program, thereby reinforcing ownership of such events and constructive response at all levels of the organization.

Inherent to the continuous quality improvement initiative is a pragmatic, systematic approach to exploring paths to improvement and error investigation rather than a reactionary, cause for blame approach to poor outcomes and lab errors. Involving members from all areas of the practice in concert with thorough and transparent data review will increase the ownership of quality across the organization. The development of a more constructive culture that seeks opportunities for quality improvement will surely realize measureable improvements in outcomes and performance in the long run.

Conclusions

An underachievement in positive clinical outcomes in ART is intolerable and demands an investigation into what factor(s) may be causing or underlying it. A well-written QC program provides not only the requirements of an optimally functioning ART laboratory but also the infrastructure for querying the performance of each element, whether technical operator, instrument, supply, or material in the effort to identify, isolate, and provide an effective remedy to it.

The potential advantage of considering causes outside the laboratory is also advanced in consideration of the multidisciplinary and multifactorial nature of ART. The use of evidence, i.e., data, elucidating outcomes stratified by both technical/laboratory and medical operators, as well as by an

array of patient demographic and clinical factors, may enhance not only the occasional need to troubleshoot in an interval of concern but to keep the ART clinic most knowledgeable about all of the facets of its practice. In driving a troubleshooting effort, leadership, collegiality, and a methodical plan are all important in its success.

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Part XIII

Special Topics

The Role of Mitochondria in the Establishment of Developmental Competence in Early Human Development

72

Jonathan Van Blerkom

Abstract

An accumulating body of evidence strongly implicates mitochondria as a central player in the establishment and maintenance of oocyte and preimplantation stage embryo developmental competence. For the human, numerical and functional defects in mitochondria have been implicated in fertilization and early developmental failure after in vitro fertilization (IVF), and as possible etiologies of female subfertility or infertility. This chapter addresses aspects of human oocyte and preimplantation embryonic development in which mitochondria appear to have critical roles. Aspects of mitochondrial function discussed include cellular bioenergetics (ATP generation), signal transduction, regulation of calcium homeostasis, and redox and reactive oxygen species-sensitive signaling. Evidence is presented indicating that abnormalities in free calcium levels in human oocytes and abnormally high levels of ATP production are associated, and may contribute to developmental abnormalities after fertilization. The finding that structurally equivalent mitochondria can be spatially distinguished in the oocyte and early embryo by detectable differences in the magnitude of the potential difference across the inner membrane ($\Delta\Psi_m$) is discussed in the context of how mitochondrial activity may be regulated, its affect on developmental competence, and whether spatial microzonation of mitochondria with high $\Delta\Psi_m$ is indicative of functional compartmentalization that is both a normal aspect of early development and one that if defective, can directly influence outcome in infertility treatments.

Keywords

Mitochondria in early human development • Genomics in reproduction • Proteomics in reproduction • Embryonic and oocyte mitochondria • Microzonation • Cytoplasmic redox state

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.

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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.

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.

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 thousands 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 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 was 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 25K 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 4,977 bp deletion (corresponding to nucleotide pairs 8,482–13,460), 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 (5,704 bp) than human (4,977 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 4,977 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 confirmed. 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 4,000 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.

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 longstanding 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.

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.

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 2-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].

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. 72.1a–d. Shortly after ionophore exposure, the relative intensity of fluorescence increases throughout the cytoplasm (Fig. 72.1b, c) before decaying to levels (Fig. 72.1d) that existed in reporter-labeled oocytes prior to activation (Fig. 72.1a). 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. 72.1e–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. 72.1e, 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. 72.1d, 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. 72.1e–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

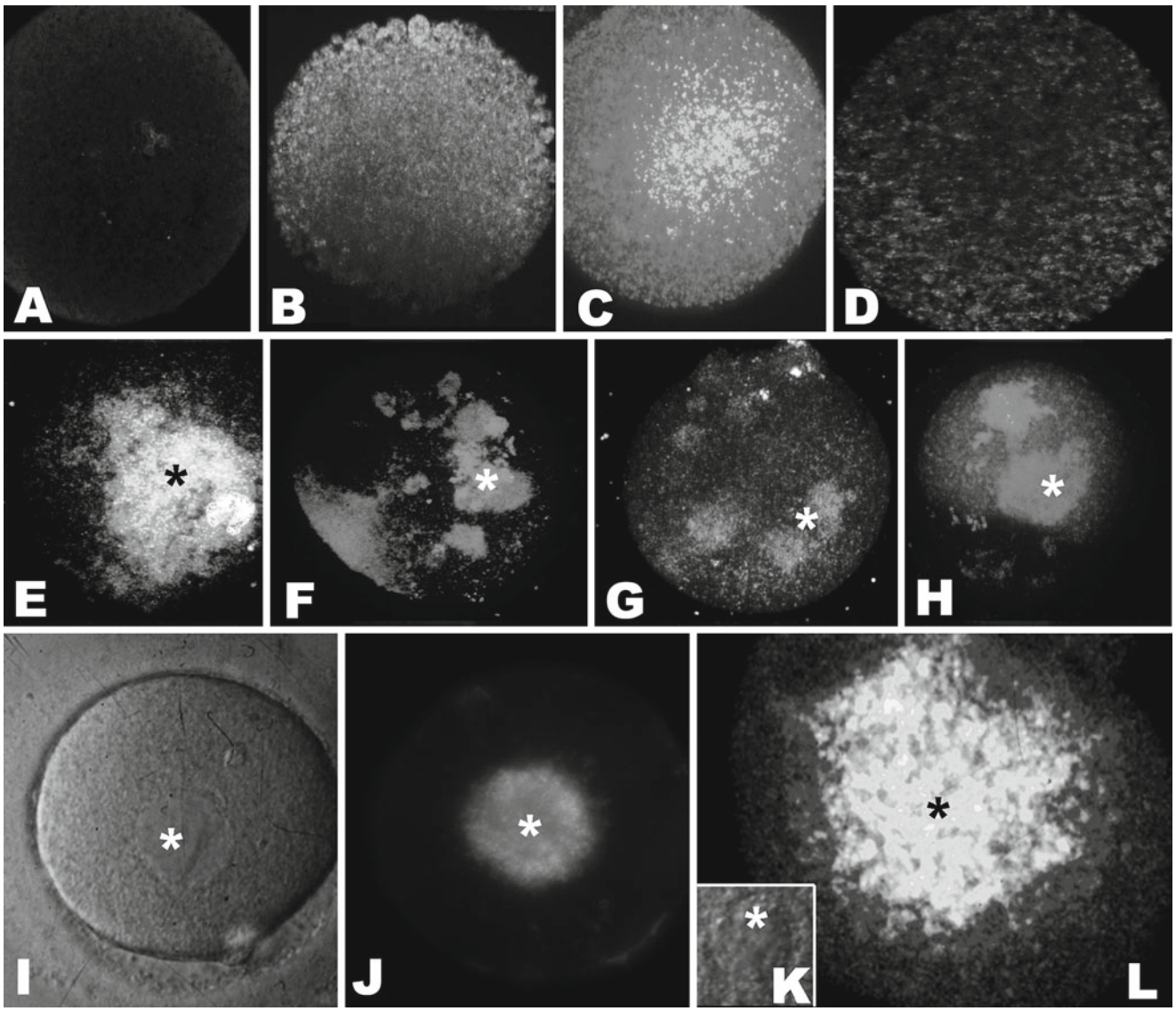

Fig. 72.1 Normal (**a**–**d**) and abnormal (**e**–**h**) patterns of increased levels of intracellular free calcium (*asterisk*) after ionophore-activation of MII human oocytes. (**j**, **l**) show a highly intense and prolonged flare of fluorescence, detected by a fluorescent calcium reporter, which 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

(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. 72.1i, 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. 72.1j) that coincides with the position of the inclusion. The intensity of fluorescence remains elevated for approximately 2–3 h (2 h, Fig. 72.1l), 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 72.2 shows selected images at

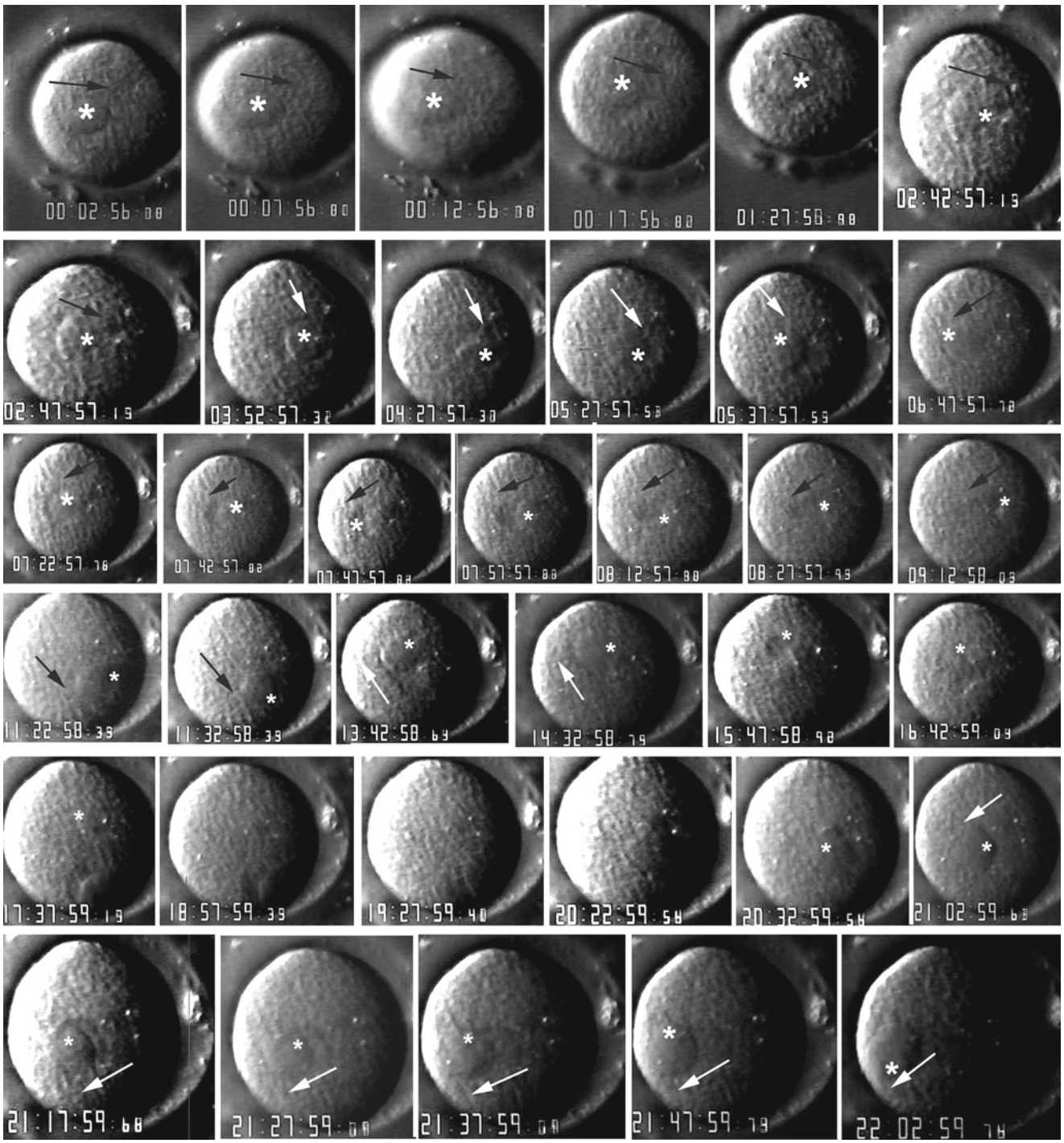

Fig. 72.2 Representative time-lapse images showing the movement of a single, larger sER inclusion (*asterisk*) in an MII human oocyte over a 22-h period. The *arrows* indicate direction at different times (indicated in *lower left*) as the inclusions move out of and into the plane of focus. This usual 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

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

to 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 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, has 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. 72.1i, 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].

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.

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 pathways. 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.

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.

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.

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Nuclear and Cytoplasmic Transfer: Human Applications and Concerns

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Josef Fulka Jr. and Helena Fulka

Abstract

The aim of this chapter is to highlight those reference 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, with modifications, in human oocytes and embryos. 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 fragmentary information we have mostly from animal experiments is clearly insufficient. An increased incidence of chromosomal abnormalities and epigenetic defects has been observed in children from oocytes injected with foreign cytoplasm, and such issues need to be resolved and further researched.

Keywords

Nuclear and cytoplasmic transfer • Oocytes in mammals • Oocyte maturation • Cytoplasmic transfer • Mutated mitochondrial DNA • Nucleolar transplantation in oocytes

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 are 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 1-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].

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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 maturation 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 normal 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.

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.

Technical Aspects

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 (cytoplast - the oocyte or zygote from which its nuclear material has been removed, enucleation) 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].

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.

Problems That Can Be Solved with Nuclear and Cytoplasmic Transfer

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].

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 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 defective 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.

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 20 h post-hCG) or possibly when oocytes are cultured in vitro, again after approximately 20 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 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 needs 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.

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, requires 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.

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 PNs-staged embryos are very resistant to damage and PNs 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., 1-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.

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.

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.

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 demonstrate 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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Cytoskeletal Architecture of Human Oocytes with Focus on Centrosomes and Their Significant Role in Fertilization

74

Heide Schatten, Vanesa Y. Rawe, and Qing-Yuan Sun

Abstract

Cytoskeletal functions are critically important for successful fertilization and for all stages of subsequent development. The present chapter is focused on the role and assessment of the centrosome–microtubule complex in the MII oocyte, in sperm, and the formation of the zygote aster after fertilization as a criterion for assessing developmental potential. We will present methods for sperm and egg quality assessment to help increase the success rate for fertilization and development, particularly when intracytoplasmic sperm injection (ICSI) is applied. ICSI is one of the current treatments to overcome male factor infertility, and it critically depends on quality assessment of sperm and egg cytoskeletal integrity. We will further propose methods to assess cytoskeletal formation in heterologous fertilization to distinguish centrosome-related male factor infertility from oocyte-factor centrosomal or regulatory defects.

Keywords

Centrosome integrity • Infertility • Fertilization • Cytoskeleton • ICSI

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 (reviewed in ref. [1]). 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 centrosomes at the opposite spindle poles by kinetochore microtubules (kMTs). Along with nucleation of kMTs from spindle pole centrosomes are pole-to-pole microtubules that are important for chromosome separation (Fig. 74.1). 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 occur during the process of IVF (reviewed in ref. [2]).

Spindle pole centrosomes 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. 74.2), 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

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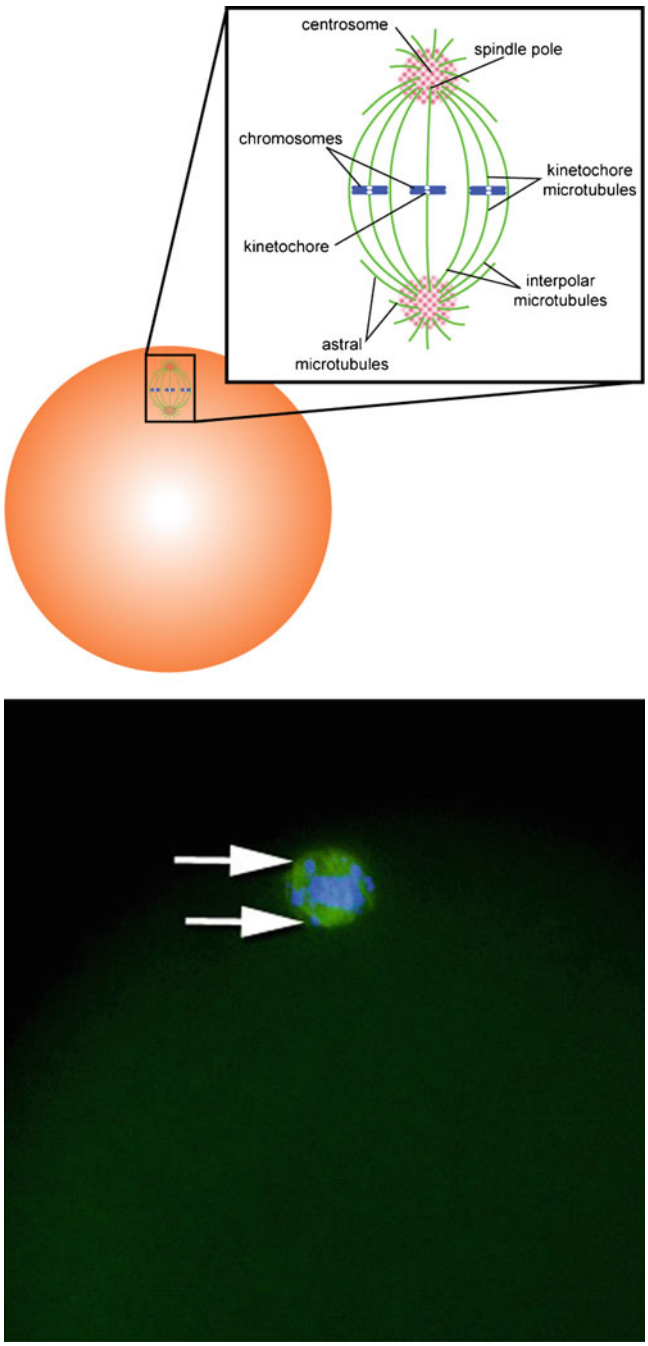

Fig. 74.1 Schematic diagram and immunocytochemistry of MII oocyte before fertilization. The MII spindle is organized from acentriolar centrosomes that nucleate kinetochore and pole-to-pole microtubules that are regulated by a complex set of kinases to hold the MII spindle in shape and prevent deterioration. 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*)

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 enormously in many aspects compared to all other mammalian species and will not be included in the present chapter.

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 refs. [3–6]). This section reviews the role of centrosomes and the MT cytoskeleton in the MII oocyte and in sperm cells.

Centrosome Proteins

The structure and composition of centrosomes has been reviewed in several previous papers [3–6] 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

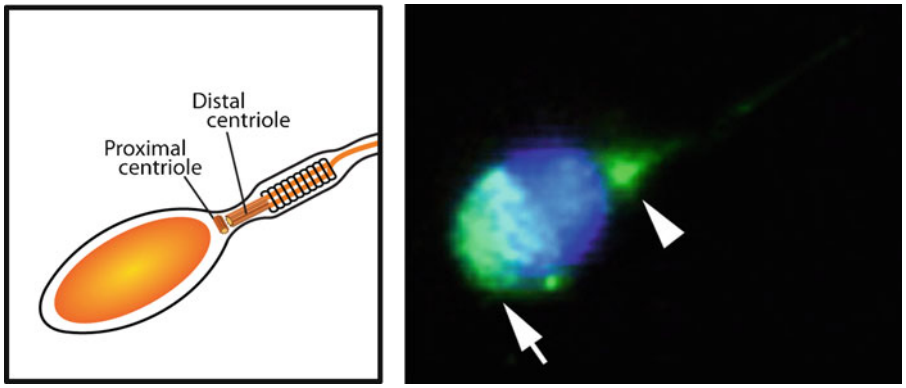

Fig. 74.2 The sperm in most mammalian species contains a proximal and a distal centriole; the proximal centriole is closely associated with the sperm nucleus and will serve as microtubule organizing center (MTOC) after fertilization while the distal centriole displays a degenerated microtubule organization 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 (from Rawe et al. [17], with permission)

as pericentriolar material (PCM). In reproductive cells, the oocyte's and the sperm's centrosomal material is reduced during gametogenesis (reviewed in ref. [7]), 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 shape. 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 [8], 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 [9]. 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.

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 refs. [7, 10–12]). During oogenesis, centrioles that are present in oogonia become lost, 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. [7]) that can be visualized in parthenogenetically activated oocytes [4, 5, 13].

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 reemphasizes the differences in mouse oocytes compared to most other mammalian species; reviewed in Schatten and Sun [4, 5]).

Although it appears static in immunofluorescence and transmission electron microscopy (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 (reviewed in Miao et al. [2]). The main functions of

the MII spindle is 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 (reviewed in Miao et al. [2]). 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. 74.1 (top panel) is an important criterion to assess oocyte quality. Misaligned chromosomes (arrow in bottom panel of Fig. 74.1) can be visualized as result of dysfunctional microtubules and molecular motor proteins associated with spindle abnormalities.

Spermatozoa

The components involved in sperm centrosomal functions have previously been reviewed in detail [4–7, 14–16]. 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 localized proximal 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. 74.2). This distal centriole becomes restructured in that it loses the triplet MT organization while a central pair of MT doublets becomes apparent, as is characteristic for the axoneme (reviewed in Schatten and Sun [4–6]). 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. 74.2; Rawe et al. [17]).

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. 74.3. 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 [17], precise nucleation of microtubules includes precise amounts of γ-TuRCs 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. 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. Overrecruitment of γ-tubulin will result in nucleation of too many microtubules while underrecruitment 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 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 [18]. 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 [19] and a variety of other centrosome-associated components that have been studied to a better degree in somatic cells (reviewed in Schatten and colleagues [3–6]). 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 [20–22]. Pericentrin forms a complex with γ-tubulin and depends on dynein for assembly onto centrosomes [22]; 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 ([23–25]; reviewed in Manandhar et al. [7]; Salisbury et al. [26]). 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

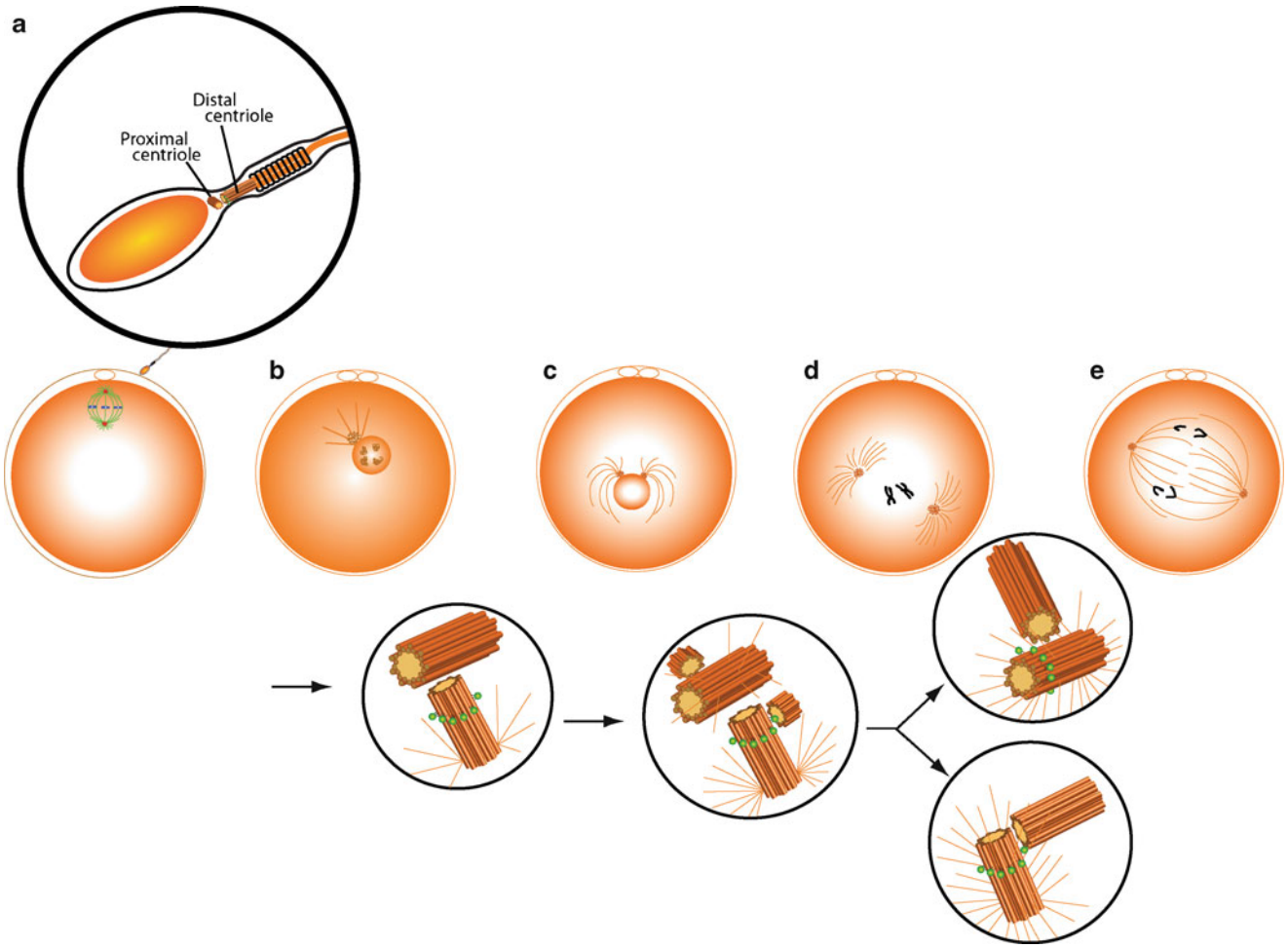

Fig. 74.3 Schematic diagram of the centriole–centrosome complex from fertilization to first cell division. (**a**) Sperm before fertilization contains a proximal and distal centriole. The meiotic spindle in the MII stage oocyte contains acentriolar centrosomes; (**b**) sperm aster formation from the 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 [6], with permission)

decondensing sperm nucleus after fertilization (Rawe et al., unpublished results). Dispersion of NuMA into the cytoplasm occurs after nuclear envelope breakdown (NEBD), followed by NuMA association with mitotic centrosomes (reviewed in Sun and Schatten [30]). NuMA is never associated with the interphase centrosome; NuMA localization in the decondensing sperm nucleus after fertilization has clearly been shown for human oocytes (Rawe et al., in preparation) and for pig oocytes [27]. 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 ([27–29]; reviewed in Sun and Schatten [30]). In aging or deteriorating oocytes, NuMA becomes dislocated from the MII spindle. NuMA abnormalities have previously been reported for cloning failures in rhesus monkeys [31].

Intracytoplasmic Sperm Injection and Assays for Centrosome Functions in ART

Intracytoplasmic sperm injection (ICSI; first reported by Palermo et al. [32]) 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 [33]), as 50% of IVF cycles are now employing ICSI in many IVF clinics. Some of the benefits using ICSI include the possibility to coinject factors that may be causes of male factor infertility problems which has already been attempted in exploratory studies using the cat as model by coinjecting 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 [18, 34]. Several heterologous ICSI systems have been employed by various investigators (reviewed in Hayasaka et al. [35]; Terada [36]) in which human sperm were microinjected into either rabbit [37, 38] or bovine [39–43] oocytes. These assays established a relationship between infertility and sperm centrosomal dysfunction [38]. Such assays have especially been useful to asses centrosome functions in globozoospermia (characterized by sperm with round heads and lack of an acrosome and acrosomal enzymes and a disorganized midpiece [44]) in which low rates of sperm aster formation was 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 [42, 45]. Figure 74.4 shows several examples of fertilization failures in human zygotes assessed by immunocytochemistry (ICC) and confocal microscopy. Studies in the domestic cat [46] 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. 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 [47]. Taken together, these studies reveal a significant role for centrosomes in fertilization indicating developmental potential.

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. [2]). While no definitely reliable noninvasive methods are available to assess spindle integrity, PolScope imaging has been applied to evaluate human oocyte MII spindle quality [48, 49]. In other test model species including the pig and bovine, immunofluorescence microscopy is typically used to evaluate spindle integrity using antitubulin 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 is shown in Fig. 74.1 bottom panel and Fig. 74.4a, respectively. It is also 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. 74.5. 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 (Rawe et al., in preparation). 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 [29].

One other aspect to assess oocyte quality for live cell noninvasive nonharmful imaging is imaging of mitochondrial distribution using multiphoton microscopy that has been employed in hamster oocytes and resulted in successful production of offspring [50]. Such approaches have not yet been used for human oocytes because of ethical considerations but 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

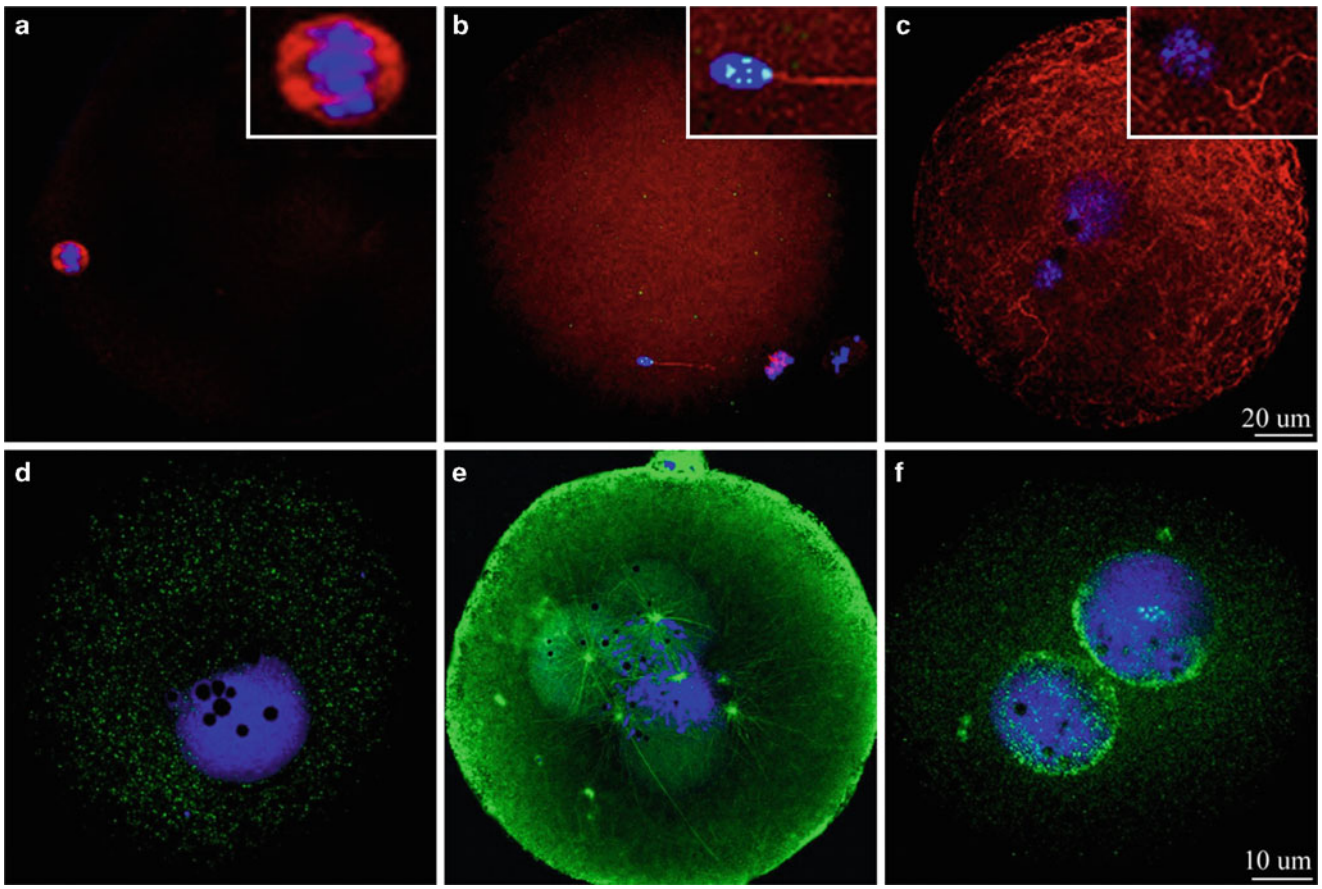

Fig. 74.4 Cytoskeletal organization and DNA configuration after failure and abnormal fertilization during IVF and ICSI. (**a**) Oocyte meiotic spindle (*red*: microtubules) with chromosomes (*blue*: DNA) at the metaphase plate. Inset in the *upper right corner* displays higher magnification detail. No 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 antitubulin 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*

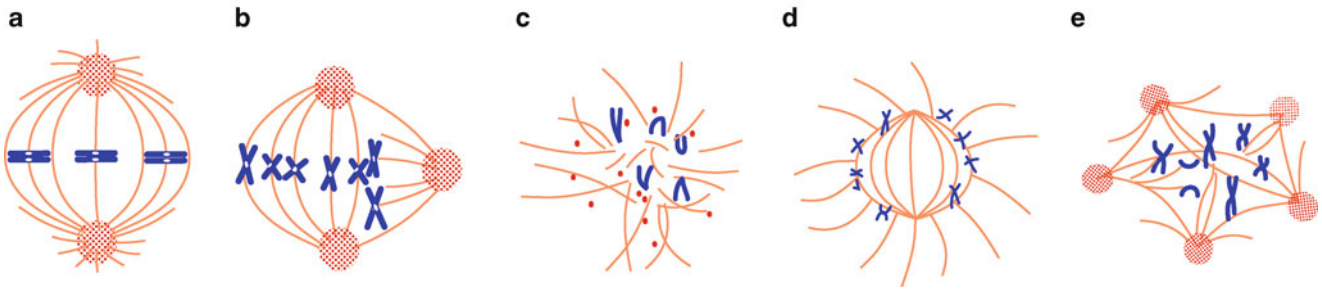

Fig. 74.5 Differences in MII spindle shapes between fresh and aged oocytes. Aged oocytes can display close to normal spindles (**a**) or highly abnormal spindles (**b**–**e**). (**b**) Tripolar spindle. (**c**) Highly disorganized spindle with scattered centrosomes and chromosomes. (**d**) Large irregular 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. [2], with permission)

localized to the basal body and can be analyzed by immunofluorescence or immunoblotting in test sperm samples. Conventional EM can easily be applied to assess sperm morphology [14, 16] 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 an 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 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. Theoretically, various regulatory factors can be coinjected 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 Schatten and Sun [4–6]).

As mentioned above, cellular organization of mitochondria has been used by Squirrell et al. [50] 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 (reviewed by Schatten et al. [51]).

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 [52]; the following sections in the present chapter are focused on centrosome detection and builds on the previous descriptions for microtubules in which media/chemicals, antibodies and staining, consumables and disposables, equipment, and tools have been described in detail. For the centrosome studies of human nonfertilized oocytes and zygotes by electron microscopy (EM), 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 [52]). Briefly, for the study of human nonfertilized 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 [52]. Examples of applying these methods are shown in Fig. 74.4.

Analysis of Sperm by Electron Microscopy and Immunofluorescence

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 Rawe and Chemes [52] in which dysplasia of the fibrous sheath is most common. Sperm abnormalities can easily be determined with 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.

To analyze sperm, a fresh sample of ejaculated sperm is centrifuged and washed in PBS for 5 min. 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 1,500–2,000 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 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%, 4 × 100%) 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 and include detailed instructions. 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 ICC* to determine the presence of cytoskeletal and centrosome components related to ultrastructure. For the ultrastructural ICC 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 is 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 is 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 cover slip on a slide warmer at 37°C. After 15 min, sperm are settled and attached to the cover slip 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, cover slips 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 cover slips 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.

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 is still needed to provide promising new therapies to possibly increase IVF and ICSI procedures related to centrosome dysfunctions.

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Molecular Mining of Follicular Fluid for Reliable Biomarkers of Human Oocyte and Embryo Developmental Competence

75

Jonathan Van Blerkom

Abstract

The occurrence of an intrafollicular biochemistry that can be used to relate oocyte developmental competence with quantitative and qualitative aspects of specific components of follicular fluid aspirated at ovum retrieval has been a goal of research in clinical IVF for decades. As a complex mixture of bioactive molecules, some produced in situ and others serum borne, there is no shortage of candidates for investigation. This chapter focuses on current methods of high-resolution biochemical analysis that can be applied to follicular biochemistry, which candidates may be the most promising in this regard, and specific investigational approaches that may have the greatest potential to fulfill this goal. In particular, emphasis is placed on whether there is a need to know both the target(s) and function(s) of candidate molecules as related to the developmental biology of the oocyte.

Keywords

Molecular mining of follicular fluid • Biomarkers of human oocyte development • Microanalytical imaging • Genetic and biochemical technologies in reproduction • Follicular fluid markers

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 developmental. 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 has 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 3 decades. Although not unexpected, the results of the chromosomal

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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 indentify 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).

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 produce 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 1,000 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, remain 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 indentified 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 imposes special requirements to assure the validity of AMH quantitation, the availability of commercial ELISA-based assays permits rapid results than 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.

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 infra red spectroscopy, NIR) can display molecular profiles or signatures of a wide array of molecules (e.g., amino acids, metabolites 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.

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 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 seems 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.

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 of 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 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 longstanding and central role of the human observer is diminished or eliminated.

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