ART: Laboratory Aspects

Charles L. Bormann

Contents

T. Falcone, W. W. Hurd (eds.), *Clinical Reproductive Medicine and Surgery*,
https://doi.org/10.1007/978-3-030-99596-6_18

Key Points

- Proper assessment, processing, and handling of gametes are essential to attain normal fertilization and high-quality embryo development.
- Many of the clinically relevant results reported from the IVF laboratory are largely based on visual morphologic assessments which can be highly subjective.
- Technologies such as preimplantation genetic testing for aneuploidy, time-lapse imaging, and artificial intelligence have been used as methods to select high-quality embryos for transfer.
- A well-established quality management plan is essential for monitoring the performance of laboratory procedures and maintaining optimal embryo culture conditions.

18.1 Introduction

The concept of IVF as a treatment for infertility is straightforward: obtain eggs from the ovaries, mix them with sperm in a dish containing culture medium, and transfer the eggs back to the woman after fertilization has occurred. However, this technique took over 100 years to develop. The initial development of IVF in humans can be attributed directly to a team of two investigators, Drs. Patrick Steptoe and Robert Edwards. It was in 1969 that Dr. Edwards first stated, "Human oocytes have been matured and fertilized by spermatozoa in vitro. There may be certain clinical and scientific uses for human eggs fertilized by this procedure" [1]. This understated conclusion marked the first successful attempt to fertilize human eggs in a laboratory.

In 1959, successful IVF was reported using rabbits [2]. The first human birth to result from IVF was achieved in England in 1978 [3]. John and Lesley Brown had 9 years of infertility secondary to bilateral fallopian tube obstruction. Dr. Patrick Steptoe surgically retrieved a single mature oocyte from one of Lesley's ovaries during a natural cycle. Dr. Robert G. Edwards combined John's sperm with the oocyte in the laboratory, and the resulting embryo was placed into Lesley's uterus a few days later. On July 25, 1978, Louise Joy Brown was delivered by cesarean section at approximately 37 weeks of gestation and weighed 5 lb., 12 oz. In 2010, 32 years later, Robert G. Edwards was awarded the Nobel Prize for Physiology or Medicine "for the development of IVF." Today, most IVF is performed after ovarian stimulation so that multiple eggs can be retrieved transvaginally with a sonographically guided needle, followed by transcervical embryo transfer.

Currently, more than 300,000 cycles of human IVF and similar techniques are performed each year in the United States, resulting in the birth of over 80,000 babies. Far-reaching advances in laboratory techniques and culture conditions have been made since 1978, when the first IVF baby was born in England. Today, ART procedures are responsible for over 2% of all children born in the United States annually [4].

Case Vignette

ART Laboratory- A 30-year-old woman with polycystic ovary syndrome has undergone IVF. She had 25 oocytes retrieved of which 5 fertilized normally and only 2 embryos made it to the blastocyst stage for transfer.

18.2 Oocyte Assessment

Mammalian oocytes are maintained in meiotic arrest throughout most of follicular development; the resumption of meiosis I (MI) is induced by the preovulatory surge of LH, which is emulated during an IVF cycle by administration of hCG. Retrieval of oocytes is generally performed 34–36 h after administration of hCG in order to allow adequate time for oocyte maturation and to avoid premature ovulation. During the oocyte retrieval, each follicle is punctured with a biopsy needle under the guidance of a transvaginal ultrasound. The fluid from within the follicle is

gently aspirated by the physician into a sterile test tube containing a processing medium. Heparin may be added to the processing medium to help prevent blood from clotting in the tube or search dishes. In the laboratory, aspirates are examined quickly for the presence of cumulus-oocyte complexes. Oocytes are rinsed thoroughly and placed in holding medium until the time of conventional insemination or cumulous cell removal.

Immature oocytes are defined as being at a stage of meiosis prior to metaphase of meiosis II (MII). This includes oocytes in prophase of meiosis I, which are identified by the presence of a germinal vesicle or nuclear envelope in the cytoplasm, without any polar body present in the perivitelline space (Fig. 18.1). If present, cumulus and corona cells are commonly very tightly condensed. As prophase I resumes, the oocyte enters into metaphase of meiosis I (MI). This intermediate stage of maturation is recognized by the disappearance of the germinal vesicle and the absence of the first polar body (Fig. 18.2). For MI oocytes, cumulus cells may be expanded, but the corona cell layer can still be compact. The extrusion of the first polar body marks the transition to a mature oocyte, which is now considered to be at MII (Fig. 18.3). Metaphase II oocytes will usually have a fully expanded cumulus cell complex. Under normal circumstances, the oocyte will remain at MII until fertilization; when meiosis II resumes, the second polar body is extruded, and the male and female pronuclei form (Fig. 18.4).

18.3 In Vitro Maturation

In vitro maturation (IVM) of oocytes is a procedure in which eggs are collected from antral follicles at a stage prior to selection and dominance. These immature oocytes are cultured under conditions that facilitate the cytoplasmic and nuclear maturation of eggs to metaphase II. This procedure is especially important for cancer patients, where the time and hormonal milieu associated with a traditional IVF cycle may adversely affect the patient's treatment and medical outcome. Likewise, patients with contraindications for

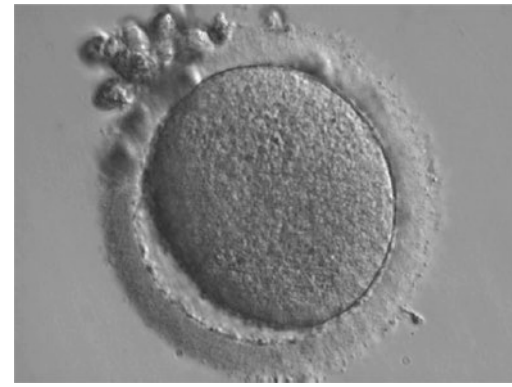

Fig. 18.2 Metaphase I (MI) human oocyte without a polar body after cumulus cell removal. (Courtesy of Dr. Nina Desai, Cleveland Clinic)

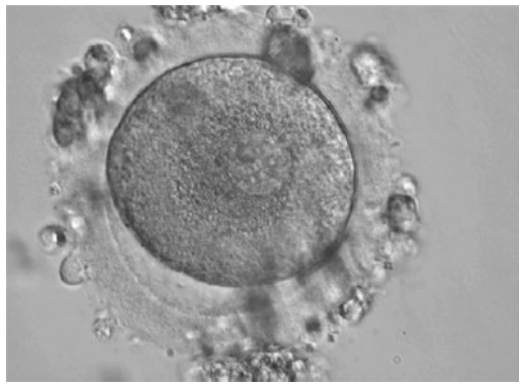

Fig. 18.1 Germinal vesicle intact representing an immature oocyte after cumulus removal. (Courtesy of Dr. Nina Desai, Cleveland Clinic)

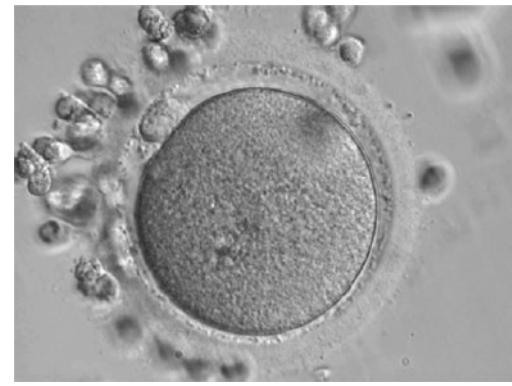

Fig. 18.3 Metaphase II (mature) oocyte. Denuded oocyte showing the presence of the first polar body. (Courtesy of Dr. Nina Desai, Cleveland Clinic)

Fig. 18.4 Normal zygote showing the presence of two polar bodies (only one seen in this view) and two pronuclei. (Courtesy of Dr. Nina Desai, Cleveland Clinic)

ovarian stimulatory drugs such as those with polycystic ovary syndrome (PCOS) and those that are at higher risk of hyperstimulation with ovulation induction agents may be candidates for IVM [5].

Although the precise mechanisms that regulate the control of oocyte maturation remain obscure, it has been recognized for over 70 years that immature oocytes removed from antral follicles may undergo spontaneous maturation in culture, termed "in vitro maturation" (IVM), without the need for hormonal stimulation. With IVM, immature oocytes are typically obtained in the mid- to late follicular phase of the menstrual cycle. To date, IVM has been most successful in young women with multiple antral follicles that typically have a high chance of pregnancy with conventional IVF. Despite this selection bias, IVM pregnancy rates remain lower than in stimulated IVF cycles [6]. As culture conditions for IVM are optimized and pregnancy rates improve, this technology may offer a safer, less expensive, more convenient alternative to stimulated IVF.

18.4 Spermatozoa Collection, Evaluation, and Processing

The most common method of obtaining a semen sample is through masturbation and ejaculation into a sterile cup. In cases where ejaculation cannot be achieved via masturbation, whether due to religious or psychological reasons, non-toxic condoms can be used to collect the ejaculation following sexual intercourse. In cases where there is no presence of an ejaculate following orgasm, patients are asked to immediately urinate in a sterile cup, and the sample is analyzed for the presence of sperm. The presence of semen in the urine is a clear indicator of a retrograde ejaculation. Men with this condition may be prescribed stomach acid buffering medications in order to neutralize the pH of the urine and thus provide a more hospitable environment for the sperm during collection and processing.

In cases where men cannot achieve an erection, or ejaculation due to neurologic or psychogenic reasons, semen can still be collected via prostate massage, electrical stimulation of the prostate, or applied vibration to the penis. Samples collected from men with spinal cord injuries typically have high concentrations of sperm and poor motility, contain red blood cell contamination in the ejaculate, and require rigorous washing steps to isolate highly motile sperm for ICSI.

In cases of non-obstructive and obstructive azoospermia where sperm are not present in the ejaculate, spermatozoa can be collected by way of testicular dissection or percutaneous needle biopsy. This method of collection is highly invasive and is generally the last resort in obtaining sperm for ICSI. Samples obtained by testicular dissection contain large amounts of red blood cells and testicular tissues; thus requiring additional steps to isolate a clean sample of spermatozoa.

18.5 Sperm Isolation for IVF and ICSI

One of the oldest and most used methods of sperm isolation is the swim-up procedure. This sperm separation technique is mostly used on normozoospermia males. The swim-up method is based on the active movement of motile sperm from a pre-washed pellet of sperm into an above layer of fresh medium. The first step in swim-up involves repeat dilution and cen-

trifugation (two to three times) of the semen sample to separate spermatozoa from seminal plasma. Following centrifugation, the pelleted spermatozoa can be both suspended and overlaid with media, or the pellet can be uninterrupted and overlaid with media. If one chooses to disrupt the pellet, extreme care must be taken when overlaying with media to prevent mixing and contamination with immotile sperm, debris, and other cell types.

The swim-up method using either the intact or disrupted sperm pellet is incubated at 37 °C for 30–60 min in a buffered media to allow spermatozoa to swim from the pellet to the culture medium. Following incubation, the upper layer of culture media is carefully aspirated without disrupting the pellet and is transferred to a clean test tube for further analysis. One advantage of this technique is that it isolates a population of sperm with greater than 90% motility and without cellular debris. The disadvantages of this technique are in the low overall recovery of motile spermatozoa due to the limited surface area of the pellet and culture media. Another disadvantage of this technique is that repeat centrifugation forces viable spermatozoa to be in close contact with immotile spermatozoa, cellular debris, and leukocytes, which are known to produce very high levels of ROS and affect subsequent fertilization ability.

Density gradient centrifugation is the second most common method for isolating motile sperm for ART purposes. Most of the density gradient systems used to isolate spermatozoa are discontinuous and consist of two to three layers. The most used materials for density gradients are colloidal silica with covalently bound silane molecules, which have a low viscosity, are non-toxic, and are approved for human use.

During centrifugation, highly motile sperm migrate faster in the direction of the sedimentation gradient and can penetrate this interface faster than low-motile or non-motile spermatozoa. This unimpeded density gradient separation produces a clean fraction of highly motile spermatozoa. The pellet is washed with culture media and centrifuged at 300 g for 10 min. This process is repeated two times to ensure complete removal of the density gradient medium prior to insemination.

There are many advantages in using a density gradient to process spermatozoa for IVF and ICSI. The entire ejaculate is used during the centrifugation process, resulting in a significantly higher yield of motile spermatozoa than can be obtained using other separation techniques. This makes this technique ideal for patients with suboptimal semen parameters (e.g., oligozoospermia and asthenozoospermia). Another advantage of this technique is that it produces a relatively clean sample of spermatozoa, free of cellular debris and leukocyte contamination. This property significantly reduces the ROS and problems associated with its contamination.

18.6 In Vitro Fertilization (IVF)

18.6.1 Conventional Insemination

Oocytes are routinely inseminated 3–6 h after oocyte retrieval is performed, depending on oocyte maturity. Individual or groups of oocytes can be incubated and inseminated either in organ culture dishes, four-well dishes, or test tubes containing equilibrated medium, with or without oil overlay. Individual oocytes can also be inseminated in 30–50-μL drops of equilibrated medium in culture dishes with oil overlay, thus reducing the number of spermatozoa necessary for the insemination. Generally, concentrations range from 50,000 to 100,000 motile spermatozoa/mL. Spermatozoa concentrations that are too high can result in increased incidence of polyspermic fertilizations (more than one spermatozoon penetrating an oocyte). Concentrations that are too low may compromise fertilization rates.

18.6.2 Intracytoplasmic Sperm Injection (ICSI)

ICSI consists of insertion of a single spermatozoon directly into the oocyte cytoplasm. This technique was first successfully applied to human oocytes in 1992 and has

since revolutionized the treatment of severe male factor infertility [7]. By injecting a spermatozoon into the oocyte cytoplasm, many steps of spermatozoa processing and developmental prerequisites are bypassed without compromising fertilization rates. There is currently a debate regarding the appropriate indications for ICSI. Current evidence supports the following indications for the use of ICSI:

- Prior failed fertilization by conventional insemination
- Prior IVF cycle with <50% fertilization of MII oocytes
- Prior IVF cycle with a high rate of polyspermic fertilization
- Total motile spermatozoa concentration less than ten million/mL
- Poor forward progressing sperm score
- Spermatozoa morphology less than 4% normal forms based on Kruger strict criteria

18.6.3 Fertilization Assessment

Fertilization assessments are performed 15–18 h after insemination for both IVF and ICSI procedures. It is necessary to examine the oocytes/zygotes within this time period to visualize the presence of pronuclei and extruded polar bodies. Normal fertilization is characterized by the presence of two pronuclei, one male and one female, in the ooplasm and two polar bodies in the perivitelline space (Fig. 18.4). If oocytes have undergone conventional IVF, the cumulus cells must be removed to clearly see the oocyte.

Abnormal fertilization may also be represented by oligopronuclear zygotes. The term applies to zygotes that have single pronuclei. Only one pronuclei and the presence of two polar bodies may be observed in cases when the oocyte undergoes parthenogenic activation or failure of the spermatozoa head to decondense. It is possible that a second pronucleus will be developed later than the first one; therefore, a repeat observation 2–4 h after the first check is recommended. Failed fertilization is represented by the absence of pronuclei and presence of one or two polar bodies that may be in the process of degeneration.

Table 18.1 Cleavage-stage embryo single-step grading system

Parameter measured	Score	Description of embryo grade
Cell number	#	Total number of blastomeres
Blastomere symmetry	1	Regular, even blastomere division
	2	<20% difference between blastomeres
	3	20–50% difference between blastomeres
	4	>50% difference between blastomeres
Fragmentation	1	<10% fragmentation of embryo
	2	10–20% fragmentation of embryo
	3	20–50% fragmentation of embryo
	4	>50% fragmentation of embryo

18.6.4 Embryo Assessment

Embryos can be assessed and graded daily while they are in culture. Standard morphologic methods of grading can be applied according to observations made on embryo development until their transfer to the uterus on day 3 (~68 h post fertilization) (Tables 18.1 and 18.2) or on day 5 or 6 at the blastocyst stage (Table 18.3) [8, 9]. There are numerous scoring systems proposed for embryo development. Criteria for grading include the rate of division as judged by the number of blastomeres, size, shape, symmetry, appearance of the cytoplasm, and presence of cytoplasmic fragments.

► Example

The grade is recorded as (cell number) C (size fragmentation); therefore, an eight-cell embryo with even cell division and approximately 15% fragmentation by volume will be scored as an "8C,1-2." ◄

Table 18.2 Cleavage-stage embryo two-step grading system

Embryo score	**Blastomere cell number**
A	Minimum of four cells by 40 h post-insemination Minimum of eight cells by 64 h post-insemination
B	Minimum of two cells by 40 h post-insemination Minimum of four cells by 64 h post-insemination
C	Minimum of two cells by 64 h post-insemination
D	No minimums (lowest possible grade). Do not make subtractions
Subtract from the grade for irregularities as follows:	
Description of embryo	**Subtractions**
Spherical blastomeres with no fragmentation	No subtractions
Spherical blastomeres with ≤20% fragmentation	Subtract one grade
Slightly irregular blastomeres with ≤50% fragmentation	Subtract two grades
Irregular blastomeres with >50% fragmentation	Subtract three grades

Table 18.3 Blastocyst-stage embryo grading system

Parameter measured	**Score**	**Description of embryo grade**
Expansion status	1	Early blastocyst; blastocoel less than half the volume of the embryo, little or no expansion in overall size, zona pellucida (ZP) still thick
	2	Blastocyst; blastocoel more than half the volume of the embryos, some expansion in overall size, ZP beginning to thin
	3	Full blastocyst; blastocoel completely fills the embryo
	4	Expanded blastocyst; blastocoel volume now larger than that of the early embryo. ZP very thin
	5	Hatching blastocyst; trophectoderm has started to herniate through the ZP
	6	Hatched blastocyst; the blastocyst has evacuated the ZP
Inner cell mass	A	ICM prominent, easily discernible, and consisting of many cells, cells compacted and tightly adhered together
	B	Cells less compacted so larger in size, cells loosely adhered together, some individual cells may be visible
	C	Very few cells visible, either compacted or loose, may be difficult to completely distinguish from trophectoderm
	D	No viable ICM cells discernible in any focal plane
Trophectoderm	A	Many small identical cells forming a continuous trophectoderm layer
	B	Fewer, larger cells, may not form a completely continuous layer
	C	Sparse cells, may be very large, very flat or appear degenerate
	D	No viable trophectoderm cells discernible in any focal plane

► Example

The grade is recorded as (expansion stage) (ICM score, trophectoderm score); therefore, an expanded blastocyst with a large tightly compacted ICM and sparse elongated Trophectoderm cells will be scored as "5A,C."

It is important to note that evaluation and scoring by morphology alone can be highly subjective and may not necessarily reveal embryos with the best developmental potential. Currently, there are numerous groups working on translational research focused on noninvasive means of assessing embryos for biomarkers that are indicative of embryonic developmental competence and implantation potential. Such evaluation, in concert with morphology and genetic analysis, has an enormous potential to reshape the practice of embryo selection in the future. ◄

18.6.5 Time-Lapse Imaging

Time-lapse imaging (TLI) of embryos was introduced in the field as an alternative method for assessing the competency of developing embryos. This technology allows embryologists to grade embryos at specific time points without disturbing the culture environment. This method of grading opened the door to using a variety of quantifiable morphokinetic measurements to aid in embryo selection. The first scientific evidence for time-lapse selection markers was discovered by a group of researchers from Stanford in 2008 [10]. They found that three cell division time intervals could predict successful development to blastocyst by day 2, the four-cell stage. The cell division timing parameters were unique, because:

1. They formed a distinct timing window where blastocysts clustered very closely compared to arrested embryos.
2. They correlated with the underlying molecular health of the embryo, as gene expression analysis showed that embryos with abnormal cell division timings had defective RNA patterns.

Since the initial identification of developmental markers for blastocyst development, numerous studies have identified a multitude of parameters that have been reportedly associated with embryo implantation potential. Some of the positive predictors of implantation include timing of compaction, timing of early blastulation, and rate of blastocoel expansion. Additionally, negative predictors of implantation have been identified using TLI. This includes the following early-stage abnormal cleavage (AC) events: AC1 (where the zygote divides to more than two daughter cells) and AC2 (where one of the daughter cells divides to more than two daughter cells). In many cases, standard morphology grading alone is unable to detect AC embryos, and these embryos are selected for freezing or transfer.

Time-lapse embryo selection algorithms (ESAs) have shown promising results in identifying embryos with low developmental potential when used by trained embryologists. The addition of TLI systems to conventional manual embryo grading has not consistently demonstrated an improvement in clinical outcomes. These systems have led to lengthier embryo morphology assessment times [11–15]. Despite the increasing use of TLI in this field for selecting embryos for transfer, there continues to be a large gap in high-quality evidence that supports its utility.

18.6.6 Assisted Hatching

One of the most common unsolved problems in IVF is the fact that embryos with apparently good developmental potential do not always implant. It has been proposed that this may be due impart to defects of the zona pellucida, uterine receptivity, extensive fragmentation, modifications after freezing and thawing, or even suboptimal culture conditions. An important observation leading to the clinical introduction of assisted hatching was the finding that there were higher implantation rates from embryos that were fertilized using microsurgical techniques such as

ICSI. In addition, it was observed that cleaved embryos with thinner zonae had higher implantation rates than those with thick zona pellucidas. It has also been reported that a naturally thick zona or hardening of the zona pellucida due to cryopreservation or suboptimal in vitro culture conditions may interfere with (and prevent) the natural hatching process, leading to implantation failure.

To overcome hatching failure, three different micromanipulation procedures (mechanical, chemical, and laser-induced hatching) have been used to thin or produce holes in the zona pellucida of cleavage-stage embryos. Assisted hatching techniques, designed to facilitate embryo escape from the zona, have been used in IVF centers since 1992. The initial indications for assisted hatching were patient age, zona thickness, high basal FSH value, and repeated IVF failure. Several retrospective and prospective studies assessing assisted hatching in these cases have given disparate results. Therefore, the clinical relevance of assisted hatching in cleavage-stage embryos within an assisted reproduction program is heavily debated [16].

18.6.7 Preimplantation Genetic Testing

Preimplantation genetic testing (PGT) includes preimplantation genetic diagnosis (PGD) performed for monogenic diseases and translocations, as well as preimplantation genetic screening (PGS) for aneuploid screening. The PGT procedure is a very early form of prenatal diagnosis for patients with a preexisting genetic risk. Technically, PGT consists of micromanipulation (biopsy) and DNA analysis of gametes and/or embryos.

18.6.8 Embryo Biopsy

Micromanipulation for the biopsy includes the mechanical opening of the zona pellucida and retrieval of one or two polar bodies (when performed on oocytes or zygotes), one or two blastomeres (when performed on cleavage-stage embryos), and five to ten cells (when performed on blastocysts). With the improvements in embryo culture, blastocyst conversion, and high success rates with blastocyst vitrification, an increasing number of labs are performing blastocyst-stage biopsy. The human blastocyst, depending upon the developmental stage, can contain more than 100 cells. As such, the biopsy of five to ten cells from the outer layer of the trophectoderm is less likely to have a detrimental effect on the developing embryo. Additional advantages of performing biopsies at the blastocyst stage include:

1. Improved development to the blastocyst stage
2. Pre-selection of top-quality embryos for biopsy
3. Improved DNA amplification with more cells biopsied
4. Lower rate of mosaicism

To aid in the biopsy of trophectoderm cells, a small opening may be made in the zona pellucida of the embryo during the cleavage stage of development. As the embryo develops and the blastocoel cavity expands, a portion of the blastocyst will herniate through the zona breach making it easily accessible for biopsy. Trophectoderm cells can be gently biopsied using a glass needle or a laser. Following biopsy, blastocysts will immediately collapse due to the opening in the zona pellucida. Blastocysts should be cryopreserved while in the collapsed state as this facilitates adequate exposure of cryoprotectants to all cells.

18.6.9 Cryopreservation

Cryopreservation of gametes and embryos maximizes success in any IVF program and prevents wastage of specimens. It is important to realize, however, that there are many ethical, religious, legal, and social implications involving embryo storage. Some countries, such as Germany, Austria, Switzerland, Denmark, and Sweden, have restricted or forbidden cryopreservation of embryos [8]. There are currently two primary categories of gamete/embryo cryopreservation strategies: slow-rate freezing and vitrification.

18.6.10 Slow-Rate Freezing

Slow-rate freeze protocols vary in permeating cryoprotectants, non-permeating cryoprotectants, and cooling and warming rates, thus making it difficult to generalize or compare cryopreservation results. The following is one general example of a cleavage-stage embryo slow-rate cryopreservation protocol.

Prior to cryopreservation, embryos that meet the program-specific freeze criteria are selected and assigned to cryopreservation. After washing embryos through processing media with 12–15 mg/mL of protein, they are exposed to the same media containing 1.5 mol/L of propylene glycol (propanediol) and then 1.5 mol/L propylene glycol plus 0.1 mol/L sucrose. Embryos are loaded into plastic straws or vials and placed in a programmable freezer, where they will be cooled at −2 °C/min from room temperature down to −4 to −6 °C. After a period of 5 min of holding the temperature, a supercooled object is pressed against the side of the container to induce "seeding." The hold is continued for a period of time, followed by continued temperature drop at a rate of −0.3 °C/min until it reaches −32 °C. At this point, the containers can be plunged directly into liquid nitrogen for storage.

18.6.11 Vitrification

Vitrification is a form of rapid cooling that utilizes very high concentrations of cryoprotectant that solidify without forming ice crystals. Ice crystals are a major cause of intracellular cryo-damage [17]. The vitrified solids contain the normal molecular and ionic distributions of the original liquid state and can be considered an extremely viscous, supercooled liquid. In this technique, oocytes or embryos are dehydrated by brief exposure to a concentrated solution of cryoprotectant before plunging the samples directly into liquid nitrogen.

Both slow-rate freezing and vitrification are being used extensively in the United States. For oocyte cryopreservation, vitrification is superior to slow-rate freezing. For cleavage-stage embryos, both approaches seem to be equally successful. For freezing at the blastocyst stage, vitrification may offer more consistent results, although slow cooling is also quite efficacious [18]. With continued research, protocols for both techniques will likely be optimized.

18.6.12 Artificial Intelligence in the IVF Laboratory

Many of the clinically relevant results reported from the IVF laboratory are largely based on visual morphology assessments. Similarly, some of the most complex laboratory procedures performed in the IVF lab, such as ICSI, assisted hatching and embryo biopsy are guided by visual morphologic cues. Manual gamete and embryo assessments are highly practice-dependent and subjective. Deep learning artificial intelligence technology has been demonstrated to help overcome the labor constraints and subjective nature of visually performing morphologic assessments in the IVF laboratory [19]. Artificial intelligence algorithms have been developed to aid embryologists with the following procedures:

- Kruger strict morphology assessments for semen analyses [20].
- Assessment of oocyte stage and quality [21, 22].
- Properly align oocytes for safe intracytoplasmic sperm injection [23].
- Perform fertilization assessment on day 1 of development [24, 25].
- Assess and select embryos for transfer at the cleavage stage of development [26].
- Identify the proper location on the zona pellucida to perform laser-assisted hatching [27].
- Assessment of blastocyst quality for PGT trophectoderm biopsy and vitrification [19, 28].
- Selection of embryos for transfer at the blastocyst stage (Fig. 18.5) [29–31].
- Prediction of embryo karyotype based on morphology [32–34].
- Cell tracking and witnessing of cleavage and blastocyst stage embryos [35].

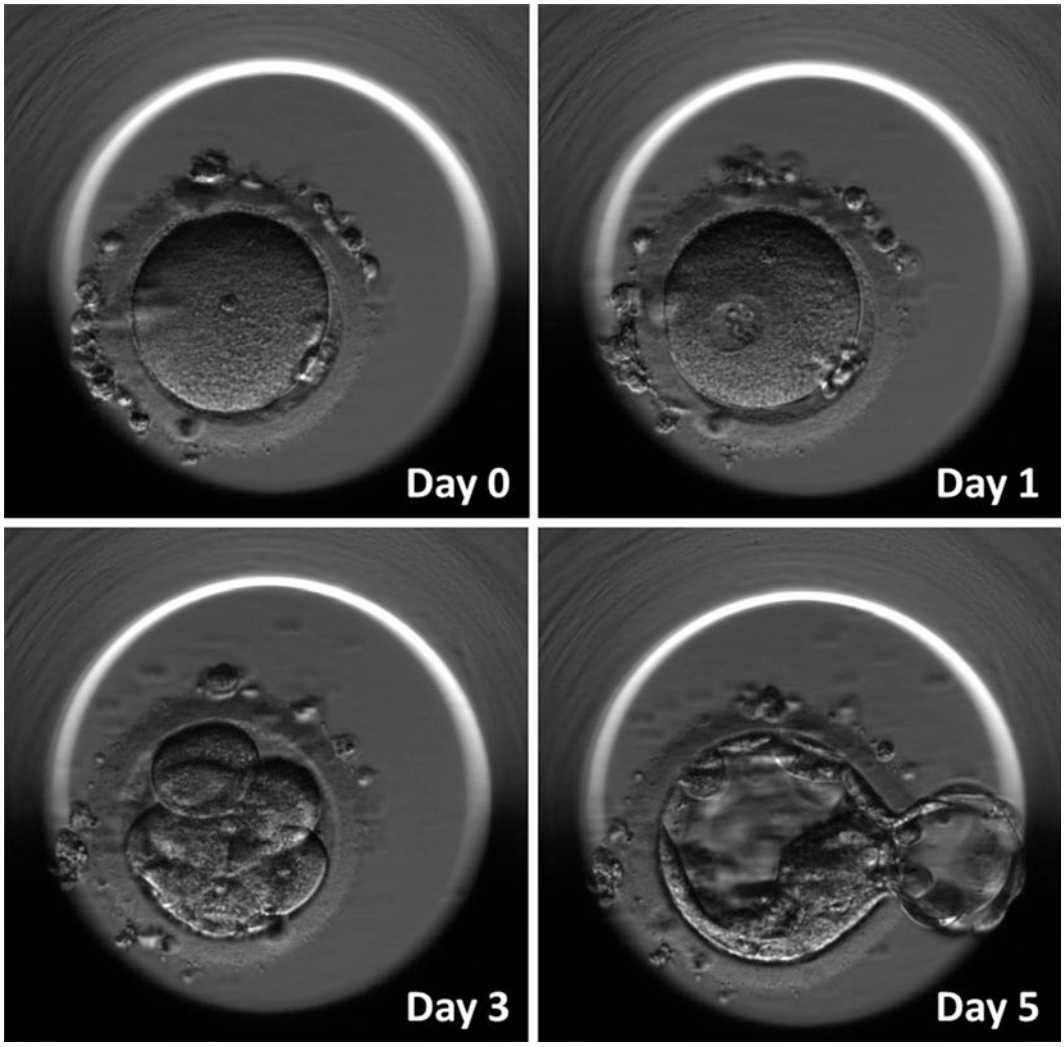

Fig. 18.5 Day 0, 1, 3, and 5 images of the first baby born that was evaluated and selected for transfer using artificial intelligence. (Courtesy of Dr. Charles L. Bormann, Massachusetts General Hospital)

- Development of key performance indicators (KPIs) to monitor laboratory performance [36].

18.6.13 Laboratory and Media Preparation

Advances in culture medium composition have significantly influenced embryo quality and pregnancy rates over the years. All media for ART can now be purchased commercially. Prior to use, they must be tested for toxins and their ability to support growth and development of embryos. Media should only be opened in a laminar flow hood, with attention to maintaining sterility during the addition of protein.

There are two basic kinds of media used for ART procedures: one used during the handling of gametes and embryos out of the CO_2 incubator, called "processing medium," and another used for culture while in CO_2 incubators, called "culture medium." Both consist of a combination of nutrients necessary to maintain early embryo metabolism and proper pH and osmolarity. A source of protein, such as albumin or synthetic serum, must be added in a percentage that can vary from 2 to 15 mg/L.

Media used for the culture of embryos are usually bicarbonate buffered and kept in an incubation chamber. It is very important that this medium is maintained at a stable pH and temperature, because embryos are extremely sensitive to variations of these two factors. Culture media, used for embryo development inside incubation chambers, should always be equilibrated inside the CO_2 environment prior to use. Overlaying of the medium with mineral oil is recommended to help avoid evaporation and increase stability of pH while the dish is temporarily out of the CO_2 environment. Oil overlay provides an effective barrier to atmospheric volatile organic compounds (VOCs), which can be embryotoxic if exposed directly to the culture medium.

18.6.14 Culture Conditions

Traditionally, laboratories have cultured embryos at an atmospheric oxygen concentration of approximately 20%. In contrast, the oxygen concentration in the fallopian tube and uterus is approximately 5% [37]. In animal models, high oxygen concentrations increase the production of reactive oxygen species. This increased oxidative stress may have deleterious effects on embryo quality [38]. Several studies suggest that culturing embryos at a lower oxygen concentration, about 5%, may improve live birth rates with IVF and ICSI [39]. Additionally, there is no evidence to date that culturing embryos under low oxygen concentrations is associated with an increased risk of any adverse outcomes, such as multiple pregnancies, miscarriages, or congenital abnormalities [39].

18.7 Monitoring Clinical Outcomes

Data analysis is a crucial part of maintaining a successful ART laboratory and practice. Routine review of identified key performance indicators (KPIs) is important to ensure proper laboratory functioning and, perhaps more importantly, to identify potential problems to permit timely correction. In fact, this is the primary reason for data analysis: to achieve early identification of factors that could negatively impact laboratory function which, in turn, allows timely insight into targets for corrective action. While KPIs may vary among laboratories, some are routinely assessed and considered standard. These include the following:

Fertilization Rates This is a useful indicator that provides real-time insight into variance in laboratory performance in addition, possibly, to changes in stimulation protocols. Fertilization rates from both standard IVF and ICSI should be evaluated with data stratified by the embryologists inseminating or injecting the oocytes.

Day 2 Cleavage The rate of embryo development is a predictive indicator of embryo quality. The number of four-cell embryos on day 2 is a common indicator of quality of the culture system.

Day 3 Embryo Development The number of cells on day 3 gives a considerable insight into performance of the lab's culture system. Embryos developing along the normal timeline should have progressed to the seven- to eight-cell stage. Therefore, the percentage of two pronuclei (2PN) zygotes with more than seven-cell embryos provides a useful marker of overall embryo quality.

Blastocyst Formation and Embryo Freezing Tracking total blastocyst formation, as well as quality of blastocysts as evidenced by those that meet a minimal freeze criterion, helps give insight into quality of the culture system. These parameters can be measured on day 5 and/or on day 6 of culture.

Cryo-survival: Tracking cell survival following cryopreservation/thawing of oocytes and embryos is an important marker of technical efficiency of a cryopreservation program. Though success rates may vary based on the stage of tissue frozen as well as method of cryopreservation (slow-rate vs. vitrification).

Pregnancy, Implantation, and Live Birth Outcomes The clinical outcome of an IVF cycle is perhaps the best indicator of system

efficiency with implantation rates providing the most robust and timely marker of embryo quality. Assessment of pregnancy rates per physician and embryologist performing the transfer is critical.

Advancements in artificial intelligence may be used to automate and improve the way we monitor laboratory performance. AI algorithms developed to assess oocyte quality can predict with high accuracy which oocytes will fertilize successfully [22]. This type of monitoring system will allow practices to monitor factors which may impact oocyte quality or suboptimal fertilization rates. AI has also been used to develop an accurate KPI to predict the developmental fate of cleavage-stage embryos. This novel KPI has been shown to have a direct correlation with ongoing pregnancy outcomes [36]. Utilization of early-stage KPIs that can predict treatment outcomes will allow us to identify and correct factors which may be altering performance within the practice and laboratory.

18.8 Future Directions

Over the past three decades, few areas of medicine have experienced the rapid evolution that has occurred within the field of ART. Despite this progress, success rates have plateaued in recent years, and many new challenges and opportunities for improvement lie ahead. With growing pressure to decrease multiple gestations, methods to improve embryo selection (such as PGS, transcriptomic/metabolomic profiling, time-lapse imaging, and artificial intelligence) will become increasingly important. Finally, as a result of these innovations within our field, we will likely see a greater shift toward eSET in all patient populations.

18.9 Review Questions

1. Which of the following are *not* indicators for performing ICSI?
   A. Prior failed fertilization by conventional insemination
   B. Sperm motility score < 50%
   C. Prior IVF cycle with <40% fertilization of MII oocytes
   D. Prior IVF cycle with a high rate of polyspermic fertilization
   E. Total motile spermatozoa concentration less than five million/mL

2. Which of the following is *not* a benefit for performing PGT biopsy at the blastocyst stage?
   A. Improved development to the blastocyst stage
   B. Higher survival rate of vitrified blastocysts
   C. Pre-selection of top-quality embryos for biopsy
   D. Improved DNA amplification with more cells biopsied
   E. Lower rate of mosaicism

3. Which of the following IVF cycle results are primarily determined based on highly subjective morphologic assessments?
   A. Sperm morphology score
   B. Oocyte maturation classification
   C. Fertilization assessment
   D. Cleavage-stage embryo selection for transfer
   E. Blastocyst-stage embryo selection for transfer
   F. Blastocyst selection for trophectoderm biopsy
   G. Blastocyst selection for cryopreservation
   H. Embryo selection for disposition
   I. All the above

4. True or false: Artificial intelligence has been demonstrated to help overcome the labor constraints and subjective nature of visually performing morphologic assessments in the IVF laboratory.
   A. True
   B. False

18.10 Answers

1. B
2. B
3. I
4. A

Acknowledgments The author would like to acknowledge the contributions of Drs. Beth Plante, Gary D. Smith, and Sandra Ann Carson who were the authors of this chapter in a previous edition.

References

1. Edwards RG, Bavister BD, Steptoe PC. Early stages of fertilization in vitro of human oocytes matured in vitro. Nature. 1969;221(5181):632–5.
2. Chang MC. Fertilization of rabbit ova in vitro. Nature. 1959;184(Suppl 7):466–7.
3. Johnson MH. Robert Edwards: the path to IVF. Reprod Biomed Online. 2011;23(2):245–62.
4. Centers for Disease Control and Prevention. 2017 assisted reproductive technology fertility clinic success rates report. Accessed 1 Oct 2020. https://www.cdc.gov/art/reports/2017/fertility-clinic.html.
5. Gremeau AS, Andreadis N, Fatum M, Craig J, Turner K, McVeigh E, et al. In vitro maturation or in vitro fertilization for women with polycystic ovaries? A case–control study of 194 treatment cycles. Fertil Steril. 2012;98(2):355–60.
6. Mikkelsen AL. Strategies in human in-vitro maturation and their clinical outcome. Reprod Biomed Online. 2005;10(5):593–9.
7. Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet. 1992;340(8810):17–8.
8. Gardner DK, Weissman A, Howles CM, Shoham Z. Textbook of assisted reproductive technologies: laboratory and clinical perspectives. 3rd ed. London: Taylor & Francis; 2008.
9. Balaban B, Yakin K, Urman B. Randomized comparison of two different blastocyst grading systems. Fertil Steril. 2006;85(3):559–63.
10. Wong CC, Loewke KE, Bossert NL, Behr B, De Jonge CJ, Baer TM, Reijo Pera RA. Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. Nat Biotechnol. 2010;28:1115–21.
11. Conaghan J, Chen AA, Willman SP, Ivani K, Chenette PE, Boostanfar R, et al. Improving embryo selection using a computer-automated time-lapse image analysis test plus day 3 morphology: results from a prospective multicenter trial. Fertil Steril. 2013;100(2):412–9.e5. https://doi.org/10.1016/j.fertnstert.2013.04.021.
12. Kirkegaard K, Ahlström A, Ingerslev HJ, Hardarson T. Choosing the best embryo by time lapse versus standard morphology. Fertil Steril. 2015;103(2):323–32. https://doi.org/10.1016/j.fertnstert.2014.11.003.
13. Kaser DJ, Bormann CL, Missmer SA, Farland LV, Ginsburg ES, Racowsky C. Eeva™ pregnancy pilot study: a randomized controlled trial of single embryo transfer (SET) on day 3 or day 5 with or without time-lapse imaging (TLI) selection. Fertil Steril. 2016;106(3) https://doi.org/10.1016/j.fertnstert.2016.07.886.
14. Chen M, Wei S, Hu J, Yuan J, Liu F. Does time-lapse imaging have favorable results for embryo incubation and selection compared with conventional methods in clinical in vitro fertilization? A meta-analysis and systematic review of randomized controlled trials. PLoS One. 2017;12(6):e0178720. https://doi.org/10.1371/journal.pone.0178720.
15. Goodman LR, Goldberg J, Falcone T, Austin C, Desai N. Does the addition of time-lapse morphokinetics in the selection of embryos for transfer improve pregnancy rates? A randomized controlled trial. Fertil Steril. 2016;105(2):275–85.
16. Practice Committee of the American Society for Reproductive Medicine and Practice Committee of the Society for Assisted Reproductive Technology. Role of assisted hatching in in vitro fertilization: a guidline. Fertil Steril. 2014;102(2):348–51.
17. Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at −196 degrees C by vitrification. Nature. 1985;313(6003):573–5.
18. Edgar DH, Gook DA. A critical appraisal of cryopreservation (slow cooling versus vitrification) of human oocytes and embryos. Hum Reprod Update. 2012;18(5):536–54.
19. Bormann CL, Thirumalaraju P, Kanakasabapathy MK, Kandula H, Souter I, Dimitriadis I, Gupta R, Pooniwala R, Shafiee H. Consistency and objectivity of automated embryo assessments using deep neural networks. Fertil Steril. 2020;113(4):781–7.
20. Thirumalaraju P, Kanakasabapathy MK, Bormann CL, Kandula H, Pavan SKS, Yarravarapu D, Shafiee H. Human sperm morphology analysis using smartphone microscopy and deep learning. Fertil Steril. 2019;112(3, Suppl):e41.
21. Targosz A, Przystałka P, Wiaderkiewicz R, Mrugacz G. Semantic segmentation of human oocyte images using deep neural networks. Biomed

Eng Online. 2021;20(1):40. https://doi.org/10.1186/s12938-021-00864-w. PMID: 33892725; PMCID: PMC8066497
22. Kanakasabapathy M, Bormann C, Thirumalaraju P, Banerjee R, Shafiee H. Improving the performance of deep convolutional neural networks (CNN) in embryology using synthetic machine-generated images. In: Human reproduction, vol. 35. Oxford: Oxford Univ Press; 2020.
23. Dickinson J, Meyer A, Kelly N, Thirumalaraju P, Kanakasabapathy M, Kartik D, Bormann C, Shafiee H. Advancement in the future automation of ICSI: use of deep convolutional neural networks (CNN) to identify precise location to inject sperm in mature human oocytes. In: Human reproduction, vol. 35. Oxford: Oxford Univ Press; 2020.
24. Dimitriadis I, Bormann CL, Kanakasabapathy MK, Thirumalaraju P, Gupta R, Pooniwala R, Souter I, Rice ST, Bhowmick P, Shafiee H. Deep convolutional neural networks (CNN) for assessment and selection of normally fertilized human embryos. Fertil Steril. 2019;112:e272.
25. Bormann CL, Curchoe CL, Thirumalaraju P, Kanakasabapathy MK, Gupta R, Pooniwala R, Kandula H, Souter I, Dimitriadis I, Shafiee H. Deep learning early warning system for embryo culture conditions and embryologist performance in the ART laboratory. J Assist Reprod Genet. 2021; https://doi.org/10.1007/s10815-021-02198-x. Epub ahead of print.
26. Kanakasabapathy MK, Thirumalaraju P, Bormann CL, Gupta R, Pooniwala R, Kandula H, Souter I, Dimitriadis I, Shafiee H. Deep learning mediated single time-point image-based prediction of embryo developmental outcome at the cleavage stage. arXiv preprint arXiv. 2020;2006.08346:5–21.
27. Kelly N, Bormann CL, Dickinson J, Meyer AD, Kanakasabapathy MK, Thirumalaraju P, Shafiee H. Future of automation: use of deep convolutional neural networks (CNN) to identify precise location to perform laser assisted hatching on human cleavage stage embryos. Fertil Steril. 2020;114(3, Suppl):e144.
28. Thirumalaraju P, Kanakasabapathy MK, Bormann CL, Gupta R, Pooniwala R, Kandula H, Souter I, Dimitriadis I, Shafiee H. Evaluation of deep convolutional neural networks in classifying human embryo images based on their morphological quality. Heliyon. 2021;7(2):E06298.
29. Bormann CL, Kanakasabapathy MK, Thirumalaraju P, Gupta R, Pooniwala R, Kandula H, Hariton E, Souter I, Dimitriadis I, Ramirez LB, Curchoe CL, Swain J, Boehnlein LM, Shafiee H. Performance of a deep learning based neural network in the selection of human blastocysts for implantation. Elife. 2020;9:E55301.
30. Chavez-Badiola A, Flores-Saiffe Farias A, Mendizabal-Ruiz G, Garcia-Sanchez R, Drakeley AJ, Garcia-Sandoval JP. Predicting pregnancy test results after embryo transfer by image feature extraction and analysis using machine learning. Sci Rep. 2020;10(1):4394. https://doi.org/10.1038/s41598-020-61357-9. PMID: 32157183; PMCID: PMC7064494
31. Tran D, Cooke S, Illingworth PJ, Gardner DK. Deep learning as a predictive tool for fetal heart pregnancy following time-lapse incubation and blastocyst transfer. Hum Reprod. 2019;34(6):1011–8. https://doi.org/10.1093/humrep/dez064. PMID: 31111884; PMCID: PMC6554189
32. Meyer A, Dickinson J, Kelly N, Kandula H, Kanakasabapathy M, Thirumalaraju P, Bormann C, Shafiee H. Can deep convolutional neural network (CNN) be used as a non-invasive method to replace Preimplantation Genetic Testing for Aneuploidy (PGT-A)? In: Human reproduction, vol. 35. Oxford: Oxford Univ Press; 2020. p. I238–8.
33. Chavez-Badiola A, Flores-Saiffe-Farías A, Mendizabal-Ruiz G, Drakeley AJ, Cohen J. Embryo Ranking Intelligent Classification Algorithm (ERICA): artificial intelligence clinical assistant predicting embryo ploidy and implantation. Reprod Biomed Online. 2020;41(4):585–93. https://doi.org/10.1016/j.rbmo.2020.07.003. Epub 2020 Jul 5
34. Chavez-Badiola A, Zhang J, Flores-Saiffe-Farías A, Mendizabal-Ruiz G, Drakeley AJ, Olcha M. Non-invasive chromosome screening and its correlation against ranking prediction made by erica, a deep-learning embryo ranking algorithm. Fertil Steril. 2020;114(3):e436–7.
35. Kanakasabapathy MK, Hammer KC, Dickinson K, Veiga C, Kelly F, Thirumalaraju P, Bormann CL, Shafiee H. Using artificial intelligence to avoid human error in identifiying embryos. Fertil Steril. 2020;113(4):e45.
36. Bormann CL, Curchoe CL, Thirumalaraju P, Kanakasabapathy MK, Gupta R, Pooniwala R, Kandula H, Souter I, Dimitriadis I, Shafiee H. Deep learning early warning system for embryo culture conditions and embryologist performance in the ART laboratory. J Assist Reprod Genet. 2021; https://doi.org/10.1007/s10815-021-02198-x.
37. Fischer B, Bavister BD. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. J Reprod Fertil. 1993;99(2):673–9.
38. Karagenc L, Sertkaya Z, Ciray N, Ulug U, Bahceci M. Impact of oxygen concentration on embryonic development of mouse zygotes. Reprod Biomed Online. 2004;9(4):409–17.
39. Bontekoe S, Mantikou E, van Wely M, Seshadri S, Repping S, Mastenbroek S. Low oxygen concentrations for embryo culture in assisted reproductive technologies. Cochrane Database Syst Rev. 2012;(7):CD008950.