CHAPTER 22

Human embryonic development in vitro

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INTRODUCTION

Human embryo culture systems have improved significantly over the past two decades. The overall increase in pregnancy rates within the USA as reported by the Centers for Disease Control (CDC) (Figure 22.1) is a direct reflection of our improved ability to culture (and select) viable human embryos. Furthermore, as well as increasing the outcome of an IVF cycle following embryo transfer at the pronuclear and cleavage stages, with the advent of more physiologic culture media, it is now also possible to culture the human embryo to the blastocyst stage as a matter of routine. This in turn has been responsible for further increases in implantation rates (and decreases in pregnancy losses). The aim of this chapter is therefore to review the current systems available to culture the human embryo and also to guide the reader to factors outside of a media bottle that can affect the outcome of an IVF cycle.

THE EMBRYO CULTURE SYSTEM

In Figure 22.2 some of the factors that can affect the outcome of an IVF cycle are highlighted. It can be seen that the embryo culture system is composed of more than the type of culture medium chosen to sustain the embryo in vitro, and that several of these factors can impact the outcome of an IVF cycle. In order to optimize outcome one must therefore consider

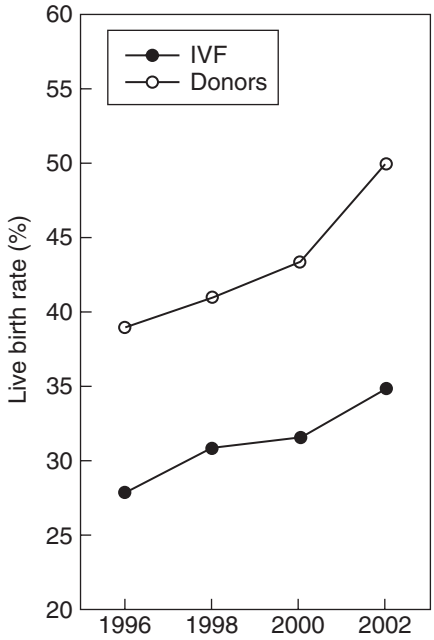

Figure 22.1 Live birth rates following IVF and oocyte donation in the USA from 1996 to 2002 (source: the Centers for Disease Control (CDC))

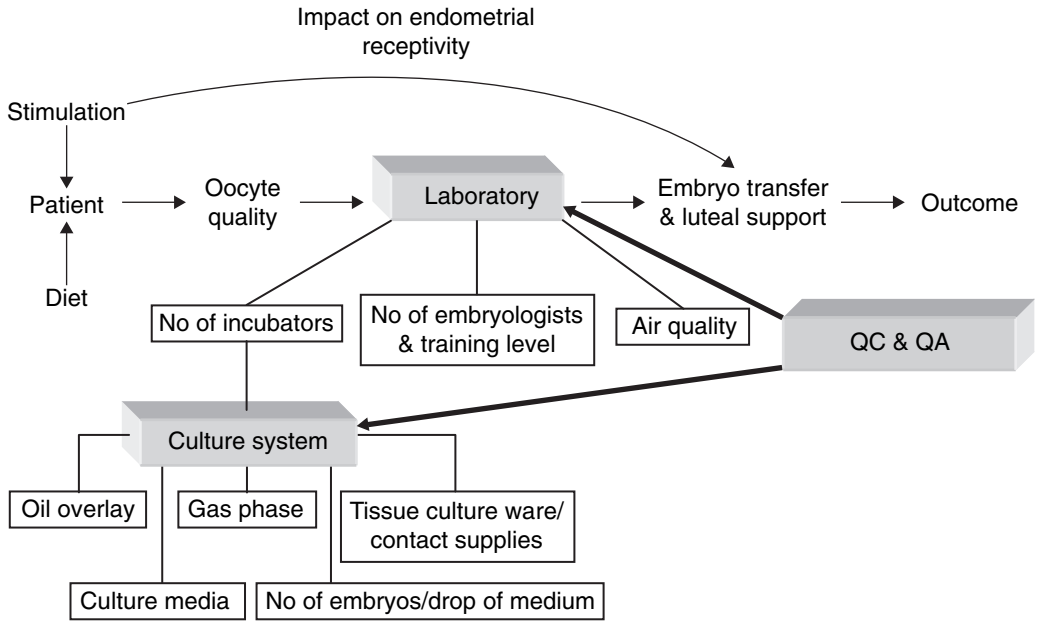

Figure 22.2 A holistic analysis of human IVF. This figure serves to illustrate the complex and interdependent nature of human IVF treatment. For example, the stimulation regimen used not only impacts oocyte quality (hence embryo physiology and viability), but can also affect subsequent endometrial receptivity. Furthermore, the health and dietary status of the patient can have a profound effect on the subsequent developmental capacity of the oocyte and embryo. The dietary status of patients attending IVF is typically not considered as a compounding variable, but growing data would indicate otherwise.

In this schematic, the laboratory has been broken down into its core components, only one of which is the culture system. The culture system has in turn been broken down into its components, only one of which is the culture medium. Therefore, it would appear rather simplistic to assume that by changing only one part of the culture system (i.e. culture medium), that one is going to mimic the results of a given laboratory or clinic.

A major determinant of the success of a laboratory and culture system is the level of quality control and quality assurance in place. For example, one should never assume that anything coming into the laboratory that has not been pretested with a relevant bioassay (e.g. mouse embryo assay) is safe merely because a previous lot has performed satisfactorily. Only a small percentage of the contact supplies and tissue culture ware used in IVF comes suitably tested. Therefore it is essential to assume that everything entering the IVF laboratory without a suitable pretest is embryo toxic until proven otherwise.

In our program, the one-cell mouse embryo assay (MEA) is employed to prescreen every lot of tissue culture ware that enters the program, i.e. plastics that are approved for tissue culture. Around 25% of all such material fails the one-cell MEA (in a simple medium lacking protein after the first 24 h). Therefore, if one does not perform quality control (QC) to this level, one in four of all contact supplies used clinically could compromise embryo development. In reality many programs cannot allocate the resources required for this level of QC, and when embryo quality is compromised in the laboratory, it is the media that are held responsible, when in fact the tissue culture ware is more often the culprit. (Modified from reference 28 with permission from Reproductive Healthcare Ltd)

each component of the culture system in turn. Subsequently, sufficient resources must also be made available for adequate quality control (QC) and quality assurance (QA), to ensure the establishment of optimum conditions within the laboratory, and the appropriate monitoring of laboratory performance.

MEDIA

It is evident that the composition of the medium used has a significant effect on embryo development and viability[1–4]. Today, unlike a decade ago, one can purchase ready to use embryo culture media from several companies. All but gone are days of in-house media preparation. One reason for this is the impact of regulatory bodies that are looking to restrict such practice. Another reason is that modern embryo culture media have increased in complexity and are therefore considerably more difficult to prepare and to ensure the highest quality. Consequently, it is important that embryologists know what media are composed of in order to make an informed decision regarding their use.

Initially, human IVF media were either those used for tissue culture (i.e. Ham's F10) or were derived from the original culture media developed for F1 mice embryos back in the 1970s[1]. Neither of the approaches can be considered optimal and, in the mid 1980s, media designed specifically for the human embryo started to appear[5,6]. Subsequently there was a resurgence of interest in mammalian embryo culture and, in the following years, several changes to media were made. One of the most significant developments in embryo culture media was the recognition that amino acids fulfill several important niches during the preimplantation developmental period. The laboratories of Gwatkin[7,8], Pedersen[9], Bavister[10], and Gardner[11,12] all determined that specific amino acids had a positive impact on embryo development, while interestingly some amino acids actually exhibited a negative effect. What also became clear was that during the preimplantation period the embryo changes its utilization of amino acids. A developing theme was that the cleavage stage embryo is stimulated by those amino acids present at high levels in oviduct fluid, while at later stages the postcompacted embryo requires a wider array of amino acids.

Amongst those amino acids found to stimulate the cleavage stage embryo were alanine, glutamate, glycine, proline, serine, and taurine. Interestingly, such amino acids are used by simple and unicellular organisms to assist in the regulation of intracellular homeostasis, such as the regulation of pH and osmolytes[13–15]. In-vitro studies on the cleavage stage mammalian embryo have revealed that such amino acids are in fact used by the embryo to regulate such intracellular functions[16,17]. Furthermore, postcompaction, when the embryo has created a transporting epithelium, such amino acids are not required to regulate intracellular function. This indicates that the embryo postcompaction is, far more robust an entity than during the cleavage stages[18].

Of general interest, those amino acids that appear at high levels within the mammalian oviduct have a striking homology to those found in Eagle's non-essential amino acids[19], defined as those amino acids not required by somatic cells in culture. Subsequently there have been numerous publications covering the non-essential group and their effects on mammalian preimplantation embryos. However, it must be pointed out that this term has nothing to do with embryology, and with hindsight perhaps a more suitable name should have been adopted. In contrast to non-essential amino acids, those termed essential (i.e. required by somatic cells in culture) cannot substitute for the non-essential amino acids prior to compaction in fulfilling these important intracellular niches[17].

Lessons learned from such work include that the oocyte, pronucleate oocyte, and

cleavage stages are dependent on specific amino acids to regulate intracellular function. It is therefore paramount that such amino acids are included in all media that the oocyte and early embryos are exposed to. Subsequently, should one wish to culture embryos to the blastocyst stage then a more comprehensive array of amino acids is required.

As with amino acids, the preimplantation mammalian embryo also changes with regard to its uptake and metabolism of carbohydrates[20,21]. Specifically, prior to compaction the embryo utilizes the carboxylic acids pyruvate and lactate as its preferred energy substrates. Glucose at this time is only consumed at low levels, and is used primarily for biosynthetic purposes and not as an energy source. By the blastocyst stage, the embryo utilizes glucose preferentially by the processes of oxidation and aerobic glycolysis. Control of nutrient uptake and utilization by the embryo is complex and changes as development proceeds[3,13,22,23]. Interestingly, in vivo the embryo is exposed to gradients of nutrients within the human female reproductive tract[24,25]. These gradients of relatively high levels of the carboxylic acids pyruvate and lactate, and low levels of glucose in the oviduct compared to the uterus, reflect the changes in carbohydrate uptakes and subsequent metabolism during preimplantation development as determined through in-vitro analysis of embryos.

Consequently, two schools of thought exist. One is that embryos should be cultured in successive media to accommodate the changes in nutrient preferences in metabolism[26]. The second is that one simply needs to provide the embryo with all the nutrients at one concentration throughout the entire preimplantation period and then let the embryo use what it requires[4]. In our experience, although the latter approach does support blastocyst development in a number of species[27], by applying a more dynamic approach to embryo culture, i.e. using media in sequence (or sequential media), one gets faster growing embryos that result in blastocysts with more cells and higher viability[28,29].

For the past decade, sequential media have been used clinically by numerous Assisted Reproductive Techniques (ART) programs. Although it can be difficult to compare data from different clinics, due to potential differences in patient populations, etc., one can derive a good indication of the efficacy of a clinic by examining their oocyte donor data. In such cases the oocytes are typically from women under 30 years old, and therefore represent a source of gametes with high viability. Oocyte donors are not only an excellent means to quantify the ability of a laboratory to culture human embryos, but they also serve as an invaluable group of patients for use in quality management. Using sequential culture media, combined with blastocyst transfer, implantation rates (fetal heart beat) of 65% can be attained, with a clinical pregnancy rate of greater than 85% when two embryos are transferred[30]. Clearly such physiologic sequential media are highly effective in a clinical setting[31].

PROBLEMS WITH STATIC CULTURE IN VITRO

Although embryo culture media have become more physiologic, for example with the inclusion of amino acids, and as a result have become more effective, one still faces the problems of working outside the female reproductive tract in a polystyrene culture dish. This can hardly be considered physiologic. This means that culture in vitro can lead to the development of artifacts that could harm the embryo. The best documented example of this is the production of ammonium from amino acids[11,32].

Although amino acids are metabolized by embryos and subsequently release ammonium in vivo, one anticipates that this ammonium is subsequently removed by the epithelial cells of the female reproductive tract, then passes through the circulation and is subsequently detoxified

by the urea cycle in the liver. However, in a static culture system any ammonium produced by embryonic metabolism simply builds up in the medium[11]. To add insult to injury, the amino acids themselves (especially glutamine) are labile at 37°C and spontaneously deaminate to release ammonium[11] (Figure 22.3). The reason that this is a cause for concern is that ammonium has been documented to affect several cellular processes in mouse and human embryos, including decreased blastocyst development, impaired inner cell mass formation, perturbed metabolism, increased apoptosis, and altered gene expression[18,32–34]. More alarmingly, if mouse embryos are transferred after exposure to ammonium in culture the resultant fetuses may be retarded, and in some cases exhibit neural tube defects[12,35].

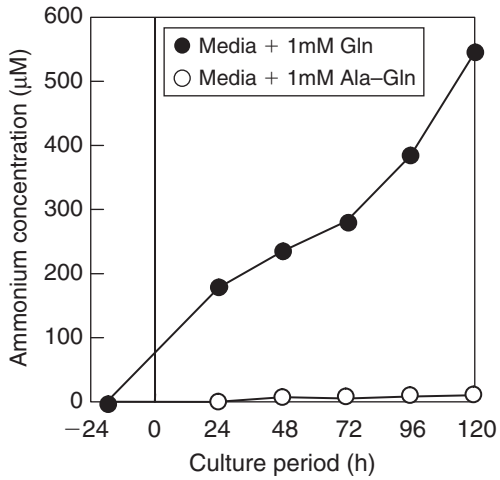

Figure 22.3 The release of ammonium into the culture medium by the deamination of amino acids. A base culture medium was supplemented with either 1 mM glutamine or 1 mM of the heat stable dipeptide alanyl-glutamine. Media were placed in the incubator at 37°C at 4:00 pm to simulate the pre-equilibration of media for use the next morning. Time zero (0 h) represents the time when an embryo would be placed in such media. However, in this example, no embryos were present. After just 48 h of culture in the presence of 1 mM GLn, embryos would be exposed to an ammonium concentration of >200 μM

Several possible remedies to this problem have been attempted, including in-situ transamination of free amino acids to glutamate using the addition of enzymes and substrates to the medium[36]. Although effective, this approach is far from practical. An alternative approach is to remove the most labile amino acid, glutamine, and replace it with a stable dipeptide form such as alanyl or glycyl glutamine[37,38]. This approach greatly decreases the release of ammonium into the medium (Figure 22.3). However, even if this is done it is still important to change the medium every 48 h as there appears to be sufficient deamination of other amino acids to produce dangerous levels of ammonium[12,38].

SUPPLEMENTS

Although embryo culture media containing amino acids do not necessarily require the addition of a macromolecule to support embryo development, embryo culture is easier and the outcome improved by supplementation with the appropriate macromolecule. The most common supplement is human serum albumin (HSA), typically in the form of albumin derived from blood. Although effective, such a source of albumin carries with it the finite possibility of disease transmission, especially in the form of prions. An alternative to blood-derived albumin is recombinant albumin. This has been shown not only to be equally as effective[39], but has the added advantage of increasing the ability of embryos to survive cryopreservation[40].

Other components of human serum documented to be effective supplement to albumin are the α and β globulins[41]. However, their efficiency has yet to be determined in prospective randomized trials. The most abundant macromolecules within the oviduct are mucins[42]. However, their

role in embryo development remains to be elucidated. Another macromolecule present within the female reproductive tract, and present at increasing levels in the uterus at the time of implantation in the mouse, is the glycosaminoglycan hyaluronan. Unlike other glycosaminoglycans, hyaluronan contains no protein moieties and can therefore be synthesized in vitro under FDA approved conditions using recombinant technology. The supplementation of hyaluronan to embryo-culture media results not only in increased cryotolerance of the embryos[40], but enhances implantation post-transfer[43]. One supplement that was popular in the early days of IVF, serum, is no longer considered favorable. The exposure of gametes and embryos to serum induces trauma, resulting in altered organelle ultrastructure, perturbed metabolism (specifically loss of oxidative capacity), altered gene expression, aberration of genomic imprinting, and ultimately altered fetal development[44–46]. It is therefore the humble opinion of this author that no gamete or embryo should be exposed to whole serum.

Another supplement to culture media for the early embryo is ethylenediaminetetraacetic acid (EDTA), which was first shown to alleviate the so called 'two-cell block' in mouse embryos cultured in a simple medium[47]. Subsequently, there has been an extensive analysis on the effects of EDTA on mouse embryo metabolism and viability[48]. The effects of 1, 10, and 100 μM EDTA at two different glucose concentrations, 0.5 and 3.15 mM (i.e. the concentrations present in the human oviduct and uterus, and in the sequential media G1 and G2), were investigated. It was determined that at 0.5 mM glucose both 10 and 100 μM EDTA significantly reduced glycolytic activity in the eight-cell embryo, but at 3.15 mM glucose the inhibition of glycolysis by EDTA was diminished. However, subsequent analysis of the glycolytic enzyme 3-phosphoglycerate kinase in two-cell, eight-cell, and blastocyst stage embryos revealed that enzyme activity was significantly reduced by 10 μM EDTA as well as by 100 μM EDTA. Given that Hewitson and Leese[49] have determined that the inner cell mass uses glycolysis exclusively, it would appear fitting to remove EDTA from the second phase media to prevent interfering with ICM physiology, which in turn could affect fetal development.

GAS PHASE, pH AND INCUBATION CHAMBERS

Embryo culture media typically utilize a bicarbonate/CO_2 buffering system to regulate the pH. This is achieved by the inclusion of 20–25 mM $NaHCO_3$ in the culture medium and the use of a 5–7% CO_2 atmosphere using a tissue culture chamber. Historically, embryo culture media are used at pH 7.4. However, analysis of the intracellular pH (pHi) of the mammalian embryo has revealed that it is actually around 7.2 to 7.3[50–52]. Therefore there has been a trend to decrease the pH of human embryo culture medium to 7.2 to 7.35.

Interestingly, the actual pH of the culture medium does not necessarily reflect that of the oocyte and the embryo[50]. Rather, by including specific amino acids in the culture medium, the embryo is able to buffer itself from changes in medium pH[17]. Furthermore, the ratios of the carboxylic acids lactate and pyruvate also impact intracellular pH[50]. However, it would appear prudent to have a medium pH of 7.2–7.35, as one does not wish to exceed a pH of 7.4. If one sets the medium pH to 7.4, then it quickly increases when the embryo's culture dish is taken out of the incubator, culminating in a rapid increase in medium pH in a bicarbonate buffer system that will affect embryonic development.

It is therefore important to monitor the pH of the medium and CO_2 environment within the incubator. The pH of a CO_2/bicarbonate buffered medium is not easy to quantitate. A pH electrode can be used, but one must be quick and the same technician must take all readings to ensure consistency. Solid-state probes are now

available with a higher degree of accuracy. An alternative approach is to take samples of medium and measure the pH with a blood-gas analyzer. A final method necessitates the presence of phenol red in the culture medium and the use of Sorensons's phosphate buffer standards. This method allows visual inspection of a medium's pH with a tube in the incubator, but is accurate to only 0.2 pH units[37]. Typically, Fyrite has been employed to quantitate CO_2 within the chamber used. However, the accuracy of such a procedure is rather low. Fortunately, there are now available new hand held infra red (IR) CO_2 analysis meters with very high accuracy (to 0.1%) (e.g. Vaisala, Helsinki, Finland).

Historically, embryo culture has been performed at ambient O_2, which is around 20%. This is in spite of the fact that the levels of O_2 in the female reproductive tract are significantly less, at only around 5–7%[53–56]. Furthermore, in several mammalian species it has been demonstrated that embryo development and subsequent viability are increased when embryos are cultured in an O_2 concentration of just 5%[57–61]. The pathologies associated with the use of high O_2 include retarded embryo development, altered metabolism, perturbed gene expression, and a changed proteome, culminating in loss of viability[18,61,62].

Some clinics have been deterred from employing a low O_2 environment because of the concerns of the cost associated with having to purchase new incubators capable of regulating both CO_2 and O_2 concentrations. However, compared to a conventional CO_2 incubator, multi-gas incubators are not that much more expensive (only around 10–20%). Furthermore, the modern multi-gas incubator uses considerably less N_2, one or two cylinders a week. Their capacity to maintain their internal environment is further enhanced by the inclusion of inner glass doors, which prevents the gas phase from being totally disrupted throughout the chamber when the main door is opened. Alternatives to multi-gas are modular chambers or even desiccators. Such chambers are purged with the gas from a mixed cylinder (e.g. 5% O_2, 6% CO_2, 89% N_2). Inside such chambers embryos are effectively isolated from the laboratory and environment within the incubator[61]. Although effective, this approach is not very practical for a busy IVF clinic. For practicality, multi-gas chambers are the preferred method. Realistically one should have two chambers per 40 to 50 patients[63], one chamber for medium equilibration in dishes or test tubes, the other chamber for gamete and embryo culture only. However, there is a role for modular chambers and desiccators in the IVF lab (see the section on quality control below). Should one go with a tissue culture incubator, ensure that the CO_2 sensor is IR and not a thermocouple type, as the latter can be responsible for too much pH drift due to fluctuations in humidity. The O_2 concentration in multi-gas chambers is regulated by the injection of N_2 to purge the ambient O_2 down to that required, typically 5–7%. Such N_2 can be supplied from N_2 cylinders, a liquid N_2 tank with an appropriate regulator, or an N_2 generator. Your choice will be influenced by the number of chambers required, which in turn is determined by patient volume.

INCUBATION VOLUME AND NUMBER OF EMBRYOS PER DROP

The optimum volume of culture medium and the number of embryos per drop has not been effectively established for the human embryo. In animal models, such as the mouse and cow, growing embryos in decreased volumes (i.e. 10–50 µl as apposed to 800 µl) and in groups (typically 4–10 embryos per drop) has been shown to stimulate embryonic development[64–67]. Hence it would appear prudent to culture human embryos in groups within decreased volumes; a typical situation is five embryos in a 40 µl drop. However, it is acknowledged that culturing groups means that a lot of information regarding embryo morphology is lost as one cannot

readily track individual embryos. Therefore, should one move to single embryo culture in order to track individual embryos, it is imperative that smaller volumes be employed, i.e. around 5 µl. However, decreasing volumes of medium results in an increase in the surface area to volume ratio of the drop, which can have significant negative impacts if there is a problem with the oil overlay. Therefore, there is an absolute requirement for oils of the highest quality.

OIL

There are numerous suppliers of oil an embryologist can choose from. The benefits of working with an oil overlay include better control of pH and osmolality by preventing evaporation of the water in the medium. Both aspects are very important. As indicated above, embryo development is enhanced in smaller volumes of culture medium. To ensure the oils used for microdrop cultures are suitable, especially at volumes between 2 and 20 µl, it is essential that such oils be tested rigorously using a suitable bioassay (see section below on quality control)[68]. Oils must be tested with a one-cell mouse embryo assay (MEA), as opposed to a two-cell test, and such oils should maintain embryos when media are used in small microliter volumes.

QUALITY CONTROL

To ensure all aspects of the culture system are optimized, it is important to use an appropriate model to screen all components individually. The MEA can be used to great effectiveness providing it is performed under conditions that maximize sensitivity. Therefore, it is important to perform such assays from the early one-cell stage, in media lacking amino acids, EDTA, and albumin (at least after the first cleavage division) and in 20% O_2. It is evident that such culture conditions are contrary to those described earlier; however, it is important to stress the embryo in order to maximize the sensitivity of the assay. Rather than a single endpoint of blastocyst development, it is important to assess development at set time points, and evaluate the blastocyst for signs of necrosis (this can be visualized easily on an inverted but not a stereomicroscope[68]). Furthermore, it is most important that the cell number of the resulting blastocysts are determined. One can readily obtain 80% blastocyst development on day 5, but it is also important to note how embryos form blastocysts in the afternoon of day 4 (i.e. on time development), and to determine the cell numbers of the resultant blastocysts is. There is a significant difference in the viability of blastocysts with 40 vs. 80 cells.

Using such an approach to testing, one can pick up subtle problems that can exist with a medium, or more typically with contact supplies. It is important to note that just because a lot number of culture dishes has been approved safe for use in somatic cell tissue culture, it does not automatically mean that it can support gametes or embryos (Figure 22.2).

With regard to determining the suitability of laboratory air, there is a quick and effective means to determine whether a problem exists in the general laboratory environment. As discussed above, the use of modular chambers or dessicators facilitates the isolation of the embryo from the rest of the laboratory. Therefore, by using a premixed cylinder, which tend to be of excellent gas quality, one can culture embryos in the IVF laboratory, but at the same time isolate the embryos from the impact of the air handling system.

HUMAN EMBRYO CULTURE PROTOCOL

The protocol described below is based on the assumption that the media in question will be renewed after 48 h. As discussed above, this is paramount whether one is using a biphasic or

non-sequential system. The protocol has been validated using the media and consumables listed. Any change to the protocol, whether it be a different source of oil or media, needs to be validated carefully. When using the sequential media G1 and G2[28], embryos should be cultured in a gas phase of 5% O_2 and 6% CO_2. Remember, human embryos will grow at 20% O_2, but development is superior at lower oxygen tensions.

Pronucleate stage embryos to day 3 culture

All manipulations of oocytes and embryos should be performed using a pulled Pasteur pipette, glass capillary, or a displacement pipette. It is important to use a pipette with the appropriate size tip. For example, once the cumulus is removed (day 1 to 3) a bore of around 275–300 μm is required. Using the appropriate size tip minimizes the volumes of culture medium moved with each embryo, which typically should be less than a microliter. Such volume manipulation is a prerequisite for successful culture.

Around 4:00 pm on the day of oocyte retrieval, label 60 mm Falcon Primaria dishes with the patient's name. Using a single-wrapped tip, first rinse the tip then place 6 × 25 μl drops of G1 into the plate. Four drops should be at the 3, 6, 9, and 12 o'clock positions (for embryo culture), the fifth and sixth drops should be in the middle of the dish (wash drops). Immediately cover the drops with 9 ml of tested oil (such as Ovoil, Vitrolife). Prepare no more than two plates at one time. Using a new tip for each drop, first rinse the tip and then add a further 25 μl of medium to each original drop. Place the dish in the incubator at 5% O_2 and 6% CO_2. Gently remove the lid of the dish and set at an angle on the side of the plate. Dishes must gas in the incubator for a minimum of 4 h (this is the minimum measured time for the medium to reach the correct pH under oil). For each patient, set up a wash dish at the same time as the culture dishes. Place 1 ml of medium G1 into the center of an organ well dish, place 2 ml of medium into the outer well and then place the dish in the incubator. If working outside an isolette use a MOPS or HEPES buffered medium with amino acids. This should not be placed in a CO_2 incubator, but rather warmed on a heated stage.

Following removal of the cumulus cells, embryos are transferred to the organ well dish and washed in the center well drop of medium in the dish. Washing involves picking up the embryo 2–3 times and moving it around within the well. Embryos should then be washed in the two center drops in the culture dish and up to five embryos placed in each drop of G1. This will result in no more than 20 embryos per dish. Return the dish to the incubator immediately. It is advisable to culture embryos in groups of at least two. Therefore, for example, for a patient with six embryos it is best to culture in two groups of three, and not four and two or five and one. On day 3, embryos can be transferred to the uterus in a hyaluronan enriched medium[43].

Day 3 embryos to the blastocyst stage

On day 3, before 8:30 am, label a 60 mm dish with the patient's name. Using a single-wrapped tip rinse the tip then place 6 × 25 µl drops of G2 onto the plate. Immediately cover with 9 ml of oil. Never prepare more than two plates at one time. Using a new tip for each drop, rinse the tip and then add a further 25 µl of medium to each original drop. Place the dish in the incubator and gently remove the lid and set on the side of the plate.

For each patient, set up one wash dish per 10 embryos. Place 1 ml of medium G2 into the center of an organ well dish. Place 2 ml of medium into the outer well. Place into the incubator. Dishes must gas in the incubator for a minimum of 4 h. If working outside an isolette use MOPS or HEPES buffered medium with amino acids. This should not be placed in a CO_2 incubator, but rather warmed on a heated stage. Set up one sorting dish before 8:30 am. Place 1 ml of

medium G2 into the center of an organ well dish. Place 2 ml of medium into the outer well. Place immediately into the incubator.

Moving embryos from G1 to G2 should occur between 10:00 am and 2:00 pm. Wash embryos in the organ well. Washing entails picking up the embryo 2–3 times and moving it around within the well. Transfer the embryos to the sorting dish and group like stage and quality embryos together. Rinse through the wash drops of medium and again place up to five embryos in each drop of G2. Return the dish to the incubator immediately. If working outside an isolette use HEPES/MOPS buffered medium with amino acids in the sorting dish. This should not be placed in a CO_2 incubator, but rather warmed on a heated stage.

On the morning of day 5, embryos should be scored (see below) and the top one or two scoring embryos selected for transfer. Manipulation of blastocysts requires the use of a capillary bore of 275–300 μm. Transfers should be performed in a hyaluronan-enriched medium[43]. Any blastocysts not transferred can be cryopreserved. Should an embryo not have formed a blastocyst by day 5 it should be cultured in a fresh drop of G2 for 24 h and assessed on day 6.

EMBRYO GRADING

Assessment of pronucleate embryos

Tesarik and Greco[69] proposed that the morphology of the pronuclei was related to the viability of human embryos. Analysis of the number and distribution of nucleolar precursor bodies (NPBs) in each pronucleus of fertilized zygotes was subsequently related to implantation potential. Features of pronucleate oocytes that had 100% implantation success were that the number of NPBs in both pronuclei never differed by more than three, and that the NPBs were always polarized or not-polarized in both pronuclei, but never polarized in one pronucleus and not in the other. Other features examined in the zygote were pronuclear orientation and size and the presence of a cytoplasmic halo. All the above features have been linked to increased pregnancy rates to certain degrees[70–72].

Assessment of cleavage stage embryos

Hardy et al.[73] showed a correlation between multi-nucleation and poor embryo development in humans. This parameter has been used in both single and double embryo transfers. Van Royen et al.[74] reported that multi-nucleated embryos had a decreased implantation rate: 5.7% in double embryo transfers. In the double embryo transfer group, two multinucleated embryos resulted in a 4.3-fold lower implantation rate than transfers of non-multi-nucleated embryos.

Another key parameter is the speed of embryo development, which can be quantitated initially at the first cleavage division. Cleavage to the two-cell stage at 24–27 h after insemination or microinjection has been shown to be a critical time point for selecting embryos for transfer[75,76]. Sakkas and colleagues have shown that early cleavage is effective in improving pregnancy rates[77]. Scott and Smith[78] devised an embryo scoring method on day 1 on the basis of alignment of pronuclei and nucleoli, the appearance of the cytoplasm, nuclear membrane breakdown, and cleavage to the two-cell stage. Patients who had an overall high embryo score (≥15) had a pregnancy and implantation rate of 34 out of 48 (71%) and 49 out of 175 (28%), respectively, compared with only 4 out of 49 (8%) and four out of 178 (2%) in the low embryo score group. Interestingly, in their study, in order to obtain a high score the embryos had to be fast cleaving; this therefore further supports the use of early cleavage as an indicator of embryonic viability.

Subsequently, Salumets et al.[79] analyzed 178 elective single embryo transfer procedures. They found that a significantly higher clinical

pregnancy rate was observed after transfer of early cleaving two-cell embryos (50%) than non-early cleaving embryos (26.4%). Van Montfoort et al.[80] have also reported a significantly higher pregnancy rate after 165 single embryo transfers of single early cleaving embryos compared with single non-early cleaving embryos (46 versus 18%). In the same study the benefit of transferring early cleaving two-cell embryos was confirmed after double embryo transfer with two early cleaving two-cell embryos as compared with two non-early cleaving embryos (45 versus 25%). In addition, the blastocyst formation of early cleaving two-cell embryos compared with non-early cleaving embryos (66 versus 40%) was significantly higher. Logistic regression showed that early cleaving two-cell embryos were an independent predictor for both pregnancy and blastocyst development in addition to cell morphology and number.

With the aim of transferring one or two embryos on day 3, Gerris and colleagues[81,82] used strict embryo criteria to select single embryos for transfer. The necessary characteristics of their 'top' quality embryos were established by retrospectively examining embryos that had very high implantation potential[82]. These top quality embryos had the following characteristics: four or five blastomeres on day 2 and at least seven blastomeres on day 3 after fertilization, absence of multinucleated blastomeres, and less than 20% fragmentation on days 2 and 3 after fertilization. Recently, the same group has reported impressive pregnancy rates after transfer of a single embryo in a selected patient population. A total of 370 single top quality embryo transfers in patients younger than 38 years of age resulted in 192 pregnancies (51.9%). Of these, 57 failed to progress and 135 (36.5%) cycles resulted in ongoing pregnancies[83].

Assessment of the blastocyst

As with the scoring of embryos during the cleavage stages, time and morphology play an important part in selecting the best blastocyst. The scoring assessment for blastocysts devised by Gardner and Schoolcraft[84] is based on the expansion state of the blastocyst and on the consistency of the inner cell mass and trophectoderm cells (Figure 22.4). Using their grading system, when two high scoring blastocysts (>3AA), i.e. expanded blastocoel with compacted inner cell mass and cohesive trophectoderm epithelium, were transferred, clinical pregnancy and implantation rates of 59/68 (86.7%) and 95/136 (69.9%) were achieved[85]. When two blastocysts not achieving these scores (<3AA) were transferred, the clinical pregnancy and implantation rates were significantly lower, 7/16 (43.8%) and 9/32 (28.1%).

The time of blastocyst formation is also crucial. When we compared cases where only day 5 and 6 frozen blastocysts were transferred compared to those frozen on or after day 7 and transferred, the pregnancy rates were 7/18 (38.9%) and 1/16 (6.2%), respectively[86]. In these cases, expanded blastocysts with a definable inner cell mass and trophectoderm were frozen. These results showed that even though blastocysts could be obtained, the crucial factor was when they became blastocysts. When taking this into account, the best blastocysts would be those that have developed by day 5.

SEQUENTIAL SCORING TECHNIQUES

By including several of the scoring criteria above, continuous scoring systems, whereby multiple parameters are used to select the best embryo, have been developed[87]. A number of these have been proposed, including one looking at day 2 and 3 assessment, using strict embryo criteria to select single embryos for transfer[81,88]. An extension of this technique was proposed by Fisch et al.[89], who used the graduated embryo score to evaluate parameters from the first 3 days of development. Finally, Neuber et al.[90] examined parameters on all 5 days of

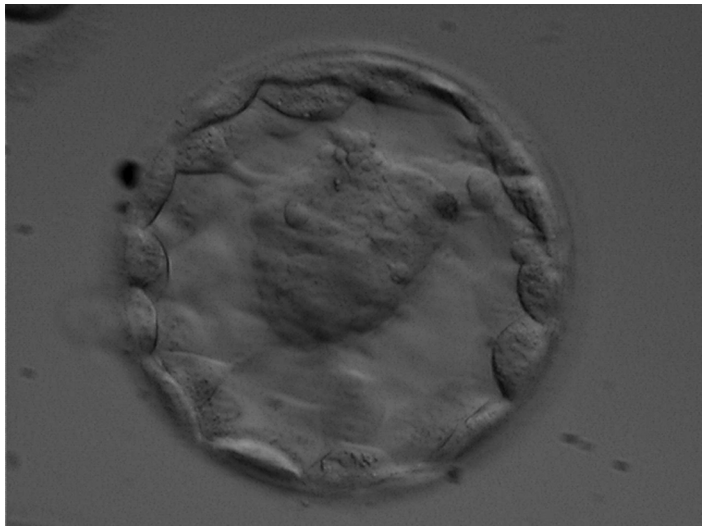

Figure 22.4 Scoring system for human blastocysts. Initially blastocysts are given a numerical score from 1 to 6 based upon their degree of expansion and hatching status:

(1) Early blastocyst; the blastocoel being less than or equal to half the volume of the embryo.
(2) Blastocyst; the blastocoel being greater than 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 herniate through the zona.
(6) Hatched blastocyst; the blastocyst has completely escaped from the zona.

The initial phase of the assessment can be performed using a dissection microscope. The second step in scoring the blastocysts should be performed under an inverted microscope. For blastocysts graded as 3 to 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 cohesive epithelium
B. Few cells forming a loose epithelium
C. Very few large cells.

The photomicrograph is of a human blastocyst (score 4AA on the morning of day 5). The blastocoel cavity is expanding and the zona is thinning (score of 4). The ICM is clearly made up of many cells (score of A). The trophectoderm can be seen to be composed of numerous cells (score of A)

development as a means of performing sequential embryo assessment using morphologically based parameters. All the above studies reported improved pregnancy rates when these techniques were utilized.

ON WHICH DAY SHOULD EMBRYO TRANSFER BE PERFORMED?

The possible benefits of blastocyst transfer are listed in Table 22.1. From such a list, it would appear that blastocyst transfer on day 5 offers several advantages over the convention of transferring early embryos on days 1 to 3 of development. However, many clinics around the world have yet to adopt extended culture and blastocyst transfer. In a review on blastocyst vs. cleavage stage transfer[91], it was noted that in most papers published on blastocyst transfer, very little information regarding the culture system, save the medium type, was ever reported. This in turn makes comparing various studies rather problematic. An analysis of 16 prospective randomized trials published to date revealed that eight studies found a positive outcome from blastocyst transfer compared to transfers on day 2 or day 3, with only one study reporting a negative outcome. The remaining seven demonstrated equivalency between days of transfer. However, more and more reports are now erring on the side of blastocyst transfer, especially with regard to single embryo transfer[92,93]. Furthermore, it appears that cryopreservation of human embryos is most effective at the blastocyst stage[94,95]. Consequently, extended culture, facilitated by the methodological advances outlined in this chapter, may represent a way to increase the overall efficiency of human IVF.

Table 22.1 Potential benefits of blastocyst transfer

- Embryo selection; ability to identify those embryos with limited, as well as those with the highest developmental potential
- Synchronization of embryonic stage with the female tract; reduces cellular stress on the embryo
- Minimize exposure of embryo to the hyperstimulated uterine environment
- Reduction in uterine contractions; reduces chance of embryo being expelled
- Ability to undertake cleavage stage embryo biopsy without the need for cryopreservation when analysis of the biopsied blastomere requires more than 24 hours
- Assessment of true embryo viability; assessing the embryo post-genome activation
- High implantation rates; reduces the need to transfer multiple embryos
- Increased ability to survive cryopreservation
- Increase in overall efficiency of IVF

CONCLUSIONS

In this chapter the significance of the media and other components of an embryo culture system have been presented. Understanding why media contain the components they do will assist the embryologist in making an informed decision as to which media to use in their own laboratory. Furthermore, by taking a more holistic look at an embryology laboratory (and clinic), one can further improve the efficacy of the culture system, leading to increased implantation and pregnancy rates.

With regards to blastocyst transfer, more information regarding the embryo can be obtained prior to transfer. This is perhaps best exemplified following in-vitro maturation (IVM), when extended culture can assist in identifying those embryos which may arrest at the eight-cell stage, due to suboptimal oocyte maturation[96].

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