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**International Scene**
Regulation of extra-embryonic development in mouse embryos
by Robert G. Edwards, PhD

**RGI Repository**
of Human Embryonic Stem Cell Lines
by Yury Verlinsky, PhD and Anver Kuliev, MD, PhD

**Vitrification:** Ready for Reproductive Medicine?
by Michael J. Tucker, PhD and Juergen Liebermann, PhD

**Assessing sHLA-G secretion:**
a useful marker for embryo selection on day 3
by Levent Keskintepe, PhD, HCLD

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International Scene

Regulation of extra-embryonic development in mouse embryos

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by Robert G. Edwards, PhD
Chief Editor, Reproductive BioMedicine Online

The initial phase of embryonic development in mice and humans involves the formation of two distinct tissues by the blastocyst stage. By this stage, embryos consist of 64 or more cells distributed between the inner cell mass (ICM), which forms the embryo proper and some placental tissues, and the outer trophectodermal layer which produces the remaining tissues of the placenta and especially cytotrophoblast and syncytiotrophoblast. These tissues are formed after hatching, as the embryo prepares for adhesion to uterine epithelium. Extra-embryonic endoderm, a derivative of ICM in mice, joins with trophectoderm derivatives in forming the differentiating placenta. Later stages involve the endoreduplication in trophectodermal/trophoblastic cells to form trophoblast giant cells. This stage is followed soon after by the formation of spongiotrophoblast and the labyrinthine layer adjacent to maternal blood vessels that is responsible for gas and nutrient exchange between mother and fetus. These essential stages prepare the placenta for its full development and roles in nourishing and protecting the developing fetus. Any errors at these critical stages can lead to placental insufficiency and fetal death.

Recent studies have begun to investigate the genetic control and characteristics of extra-embryonic tissues in implanting mouse embryos. Numerous studies have utilized genetic manipulations, such as knockout of specific genes, to study the roles of retinoblastoma (Rb) and Dp1 in mouse embryogenesis, and the effects of gene modifications on continued growth of embryonic and extra-embryonic tissues. Two independent studies have carried this work much further. Each has revealed highly interesting and significant aspects of growth during these stages by providing detailed analyses on results of knockout on the initiation and development of extra-embryonic tissue, with some surprising embryonic and physiological effects on early-mid-pregnancy.

Dp1 and E2F1 facilitate or suppress the progression of the cell cycle. Dp family members work in unison with E2f1, Dp1 being an absolute requirement for embryo survival (Kohn et al., 2003). Knockouts of E2F produce mild phenotypic changes in the embryo. In contrast, knockout of Dp1 leads to the death of the embryo in utero because of the failure of extra-embryonic tissues to develop normally. Fetuses in Dp1−/− mice die at gestation day 12.5 with extra-embryonic defects in trophectoderm derivatives including fewer trophoblast giant cells, fewer cells in ectoplacental cone and chorion, and reductions in amounts of DNA in extra-embryonic tissues which impose growth retardation in embryos.

Dp1−/− acts by compromising the lineage-specific formation and growth of tissues derived from trophectoderm, especially the expansion of ectoplacental cone and chorion, and the onset of endoreduplication in trophoblast giant cells. There is thus an absolute requirement for Dp1 for extra-embryonic development and fetal survival, consistent with the promotion of normal growth by E2F/DP1. The gene may have other as yet unknown effects, although apoptosis regulated by p53 is not among them. Mutations in E2f1 or E2f3 share the effects of Rb−/− and their actions become more specific to particular tissues. These mutations also produce a disposition to tumours (Kohn et al., 2003).

Retinoblastoma was the first reported tumour suppressor gene. The Rb gene is again closely associated with the heterodimers E2f1/DP1 in mice which are released from repression by the retinoblastoma tumour suppressor (pRB) to permit cells to enter their S phase. Various family members affect cell mortalization and cell cycle checkpoints. Its inactivation leads to unscheduled cell proliferation, apoptosis and widespread developmental defects. In knockout Rb−/− mice, fetuses die in utero at ~15 days, i.e. in mid–late gestation. They display deficits in the cell cycle, and numerous anomalies including severe weaknesses in their overall differentiation of central nervous system, liver, brain etc., which were believed to be consequential on the inheritance of a mutated gene (Wu et al., 2003). Close analysis of Rb−/− pregnancies revealed an excess proliferation of trophoblast stem cells had reduced placental vascular transfer by decreasing blood spaces and reducing blood flow. Direct effects were seen as clusters of small cuboidal trophoblasts lined maternal blood spaces, elongating them and so reducing their surface area by 40%. Rates of apoptosis in the labyrinth were higher than in controls, and lower levels of fatty acids in fetuses indicated the onset of poor placental transport. It is also possible that the control of some checkpoints during mitotic cycles has been lost.

Since placental insufficiency was a characteristic of
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In contrast to conventional cryopreservation, vitrification is an ice-crystal-free cooling method for biological materials. Especially chill-sensitive cells in ART like oocytes and blastocysts have a big advantage in survival rates by avoiding ice-crySTALLization using vitrification. The physical definition of vitrification is the solidification of a solution at low temperature, not by ice-crySTALLization but by extreme elevation in viscosity during cooling. The conditions necessary to achieve this are a high cooling rate in the range of minus 15,000 to 30,000°C/min to transform water directly from the liquid phase to a glassy vitrified state; and a relatively high concentration of cryoprotectant is also required. While the terms “freezing” and “thawing” are commonly used for conventional cryopreservation, the terms “vitrifying” and “warming” should be used for vitrification procedures. Vitrification is a very simple, fast and cheap method. No special equipment is necessary as the cells and tissues are placed into the cryoprotectant, and then plunged directly into liquid nitrogen. The cryoprotectant largely replaces the intracellular water during this process.

It is important in vitrification to use special carriers to keep the sample volume as small as possible, and thereby achieve as rapid a cooling rate as possible. Many carrier systems have been described to date such as open-pulled straws, flexiPET denuding pipettes, microscope copper grids, cryoloops, and the hemi-straw system, all of which aim to minimize the volume to be vitrified to <5 microliters.

Vitrification in Assisted Reproductive Technology has been described so far for gametes and embryos (all developmental stages from zygote to blastocyst), as well as for ovarian tissue. Spermatozoa are usually frozen by conventional cryopreservation, because the high concentration of cryoprotectant in vitrification affects the sperm motility and may have also a negative genetic influence. Nevertheless successful vitrification of human spermatozoa has been reported. Cleavage-stage embryos can be frozen either by conventional cryopreservation or by vitrification with similar survival rates; however there is considerably more experience using conventional cryopreservation. There are very few data available concerning the vitrification of human ovarian tissue. Due to the large tissue size it is difficult to maintain the high cooling rate, and therefore there are problems with fracturing of the tissue and crystallization. As mentioned previously, the greatest advantage of vitrification has been seen with chill-sensitive cells like oocytes and blastocysts.

The oocyte is extremely sensitive to ice-crySTALL formation. Various types of cell-injuries like damage to intracellular lipid droplets and the cytoskeleton can occur. Furthermore the sensitivity to osmotic swelling, which can occur during the removal of cryoprotectant from cryopreserved cells, is very high. This can be explained by the decreased permeability of the cytoplasmic membranes of oocytes. The more mature the oocyte is, the more sensitive it is. After initially inconsistent results with oocyte vitrification, more recent results are very
promising. In particular, after changing the standard cryoprotectant from dimethyl sulphoxide (DMSO) and 1,2-propranolol to a combination of ethylene glycol (EG) and DMSO, the cryo-survival rate of vitrified oocytes increased to more than 70%, and even to 90% in studies with more limited oocyte populations. Two unpublished examples of oocyte vitrification help to support the above statement. In a case at the Fertility Centers of Illinois (Chicago) 17 oocytes were vitrified when no spermatozoa were available on the day of retrieval. About three month later, all oocytes were warmed, 13 survived (survival rate of 77%) and were injected; 12 of them fertilized normally (fertilization rate of 92%). All of them developed (cleavage rate of 100%), and on day-3 there were six 8-cell, three 6-cell and three 4-cell embryos available for transfer. Four 8-cell grade-1 embryos were chosen and transferred. Two blastocysts were re-vitrified on day-5, and three blastocysts were re-vitrified on day-6. Two weeks later, the pregnancy test was positive and the hCG increased up to 2,500. Unfortunately this pregnancy resulted in an early miscarriage. In a donor oocyte cycle at the Mississippi Fertility Institute (Jackson) 11 surplus oocytes were vitrified. In the first vitrification cycle five out of six oocytes survived (83%), two out of five fertilized normally (40%), and ultimately one 7-cell embryo was transferred; the pregnancy test was negative. In a second cycle two out of five oocytes survived (40%), these two oocytes were injected with sperm, and one fertilized (50%). On day-3 one 8-cell embryo was transferred. Unfortunately the pregnancy test was initially positive, but eventually did not continue successfully. While these results are very limited, there are numerous successful reports in the literature of oocyte vitrification, with good development to the blastocyst stage, pregnancies and live births. Both programs referred to above have adopted vitrification as their standard cryopreservation approach for blastocyst cryostorage with very encouraging clinical results to date.

The blastocyst stage embryo is characterized by a fluid-filled cavity called the blastocoel. It seems that with increasing volume of the blastocoelic cavity, the survival rate drops with vitrification. This is probably due to insufficient permeation of cryoprotectant into the blastocoel, so that residual water may cause ice-crystallization during the vitrification process. Recently, publications have shown that survival rates of cryopreserved expanded blastocysts can be improved by artificial reduction of the blastocoelic cavity. Comparing the survival rate of rhesus monkey blastocysts from slow-freezing protocols and vitrification protocols, data have shown a post-thaw survival rate of 36% with 5% hatching after slow freezing and of 85% with 71% hatching after vitrification. Additionally, after vitrification of human blastocysts using the cryoloop as carrier survival rates of 72-90%, clinical pregnancy rates of 37-48%, and an implantation rate of 22-29% has been reported. At the Fertility Centers of Illinois (Chicago) we have experience with both slow freezing and vitrification of blastocysts, and have seen to date a pregnancy rate of 49.5% (45 pregnancies/91 thawed embryo transfers) after slow freezing of blastocysts; and 54.5% (18 pregnancies/33 warmed embryo transfers) after vitrification of blastocysts (unpublished).

In summary, vitrification is a very promising cryopreservation method. The main advantages compared to slow-freezing protocols are the better survival rates with both oocytes and blastocysts, as well as the shorter procedure time without the need of special equipment.

Suggestions for Further Reading


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In a series of articles we will explore the clinical and laboratory aspects of the most common diagnoses that would lead to consultation with a Reproductive Endocrinologist for infertility. Our goal is to provide the most current clinical and laboratory aspects associated with treatment for unexplained infertility, polycystic ovarian syndrome, recurrent pregnancy loss and severe male factor, and to explore the treatment modalities available. We will present the information in a manner adaptable for couples undergoing treatment rather than the professionals involved in providing it. In the current issue we will address a couple with unexplained infertility.

Lisa and her husband Jim tried unsuccessfully for 12 months to conceive a child and were referred to a Reproductive Endocrinologist for consultation. Lisa is a 28-year-old with normal ovulatory cycles. Her initial evaluation revealed a normal anatomical (structural) assessment, and normal endocrine/hormonal function. Jim’s semen analysis was well within normal limits. Lisa and Jim agreed to commence their treatment with ovulation induction cycles in combination with intrauterine insemination using clomiphene citrate, an oral medication that stimulates multiple follicles, thus multiple oocyte development. After 3 failed cycles, Lisa consented to undergo an exploratory laparoscopy during which stage II endometriosis (staged I through IV, with IV being the most advanced) was identified and treated. The tubal and uterine anatomies were confirmed as normal. Although endometriosis is causal for subfertility (not infertility), Lisa and Jim were advised to commence a more aggressive strategy of ovulation induction with the injectible medication, Gonal-F. Although Lisa responded well to the stimulation regimen, with the appropriate ovarian response, she failed to achieve a pregnancy.

Lisa and Jim were counseled regarding undergoing in vitro fertilization (IVF) due to the previous failed interventions. Failure to conceive with either level of ovulation induction management may suggest fertilization issues, mechanical or physiological, which really can only be assessed via IVF. Lisa and Jim agreed with this course of treatment. Controlled ovarian hyperstimulation was initiated; Lisa responded well to her individualized regimen with >10 follicles visualized on ultrasound and a serum estradiol level (an index of follicular growth) appropriate for the swift response.

The date of Lisa’s oocyte retrieval was approaching. During preparation for their IVF cycle, Lisa and Jim viewed our various patient handouts and spent many hours on the Internet expanding their IVF knowledge. Nonetheless, this preparation did not appear to relieve the trepidation and queries their cycle introduced. To assist you in understanding the information that your clinical or laboratory staff may communicate during your IVF-ET cycle, we have addressed many of Lisa and Jim’s questions. We strongly believe that our patients should be well informed during every aspect of their treatment and communicate daily to ensure a complete understanding of the daily progress within the laboratory.
What happens after the oocyte retrieval and will we need intracytoplasmic sperm injection (ICSI)?

The day of oocyte retrieval is known as Day 0 in the laboratory. Jim will collect his sample in the morning while you are in surgery. The sample will be processed to concentrate a highly motile, or active, fraction for insemination. After all of the oocytes are identified, they are stored in the incubator until it is time for insemination (~4-6 hours after the retrieval). The parameter's of Jim's sample are all well above the norm, therefore, we anticipate that we will use conventional insemination to achieve fertilization. Although it is a program-specific preference when to employ ICSI, it is a procedure that was intended for use with compromised semen specimens. With conventional insemination, a predetermined concentration of sperm is added to the oocytes to initiate fertilization. This method was inadequate for semen samples with any compromised parameters, so the ICSI procedure evolved. This process, when a single sperm is injected into a single oocyte, was a significant advancement in the treatment of male factor infertility. Although the end result of the two methods is the same, the procedures vary significantly in complexity.

How and when do you assess fertilization?

Approximately 15-18 hours after insemination, the oocytes are assessed for fertilization (on day 1). At this stage the pre-embryo is characterized as a single cell with two centrally located pronuclei (designated as a 2pn, figure 1). The pronuclei represent the nuclear material of sperm and the oocyte. They are maintained as separate entities within the oocyte at this time but will shortly merge to create a unique genome. The 2pn morphology can offer some insight as to the potential growth of the embryo; therefore, a rigid assessment is documented.

When do the embryos progress beyond the 1 cell stage?

Growth (division) of the embryo is assessed on days 2-5. Days 2 and 3 are characterized by relatively slower embryonic growth so that the embryo divides to ~6-8 cells by the morning of day 3 (figure 2). Divisions from this point occur at a more rapid pace so that by mid-afternoon of day 5, the embryo is now a blastocyst composed of approximately 60 cells (figure 3). The cleavage or growth assessments can provide some critical information in selecting the embryo(s) for intrauterine transfer. On days 2 and 3 embryos with the greatest growth potential are those with symmetrical, smooth cells containing a single nucleus and little to no fragmentation (breakdown of cells). An embryo with a very defined spatial arrangement such as in figure 2 is also more desirable for embryo transfer. What you will find is that throughout the culture period, the number of embryos available for transfer will decrease over time, as not all embryos share the same capacity for development.

When is our embryo transfer and how many embryos will be transferred?

The developmental stage selected for embryo transfer is generally a program based decision and is typically days 3 or 5, with some programs still adhering to day 2 transfers. There are no regulations in the US, but merely guidelines, governing the quantity of embryos transferred to the uterus. In general the number for transfer depends on a several factors, including but not limited to, patient age and history, embryo quality and sperm quality. Our approach regarding the day of transfer and the number to transfer is individualized on a per patient basis and includes input from the laboratory, physician and patient/patient couple. Also, the day of embryo transfer significantly influences the number of embryos. The earlier the stage of development, such as day 3, the more aggressive we are at ET. For instance on day 3 we might transfer 2-4 embryos depending on the aforementioned criteria, but typically our day 5 transfer does not exceed 2 embryos. Embryos that have grown to blastocyst on day 5 have a higher rate of implantation not only because they have maintained their growth in the culture system, but because they are at the developmentally appropriate stage normally present in the uterus during a natural cycle.
How do these cycle characteristics relate to the chances of achieving pregnancy?

The clinical and laboratory aspects of the IVF cycle are key indicators for pregnancy rate. The ovarian response to the stimulation certainly influences the oocyte and thus embryo quality. In addition, the sperm possesses a vital function in not only fertilization but in growth and development of the embryo. Therefore, there is a collective role in follicular response and development, and oocyte and sperm qualities in not only the implantation rate but also the live birth rate. Equivalent in importance in achieving a pregnancy is the uterine environment, or more specifically, the receptivity towards the growing embryo. This not only involves an apposite thickness, as measured by ultrasound during your cycle management, but also the presence of surface moieties involved with embryo recognition and binding. In essence, many factors both embryo and uterine derived contribute to the implantation and pregnancy rate.

In the subsequent issue we will discuss the clinical and laboratory aspects of patients with polycystic ovarian syndrome, including management during the IVF cycle.

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Figure 1: A fertilized oocyte with 2 pronuclei (2pn) as observed on day 1 of culture.

Figure 2: An 8-cell embryo as observed on day 3 of culture.

Figure 3: A blastocyst as observed on day 5 of culture.
We Must Not Forget

by Liz Sanders, Chief Embryologist
Mississippi Fertility Institute

It is 10:00 p.m. Monday night and we are getting ready to retire for the evening. Our thoughts are focused on what we will wear tomorrow, what errand we might need to run after work, did we fix the coffee pot, getting our kids ready for bed and probably the weather.

Somewhere close by is a couple filled with excitement, probably too much so to sleep, about tomorrow. They are focused on getting to the doctor’s office on time and most of all on what is REALLY going to happen.

You see, this couple has no children and their only hope of achieving a pregnancy is In Vitro Fertilization. They have come to our facility for help.

The wife has struggled through the daily injections, mood swings, vaginal ultra sounds, weight gain, numerous blood draws, seen many different faces and I’m sure many other emotions. Likewise, her husband has had his own trials to endure. YEP! Waiting in a waiting room filled with women waiting for his name to be called to submit his semen sample or better yet, to be taken to unfamiliar territory to collect his sample. Oh Joy! Through it all he is a supportive spouse and fulfills his part. Have we forgotten all of this?

As an IVF team we should all remember our patients are real people with real feelings and real emotions. Listening, answering questions and supplying information are other human elements we fulfill for the patients.

As an IVF team we should all strive to treat the patients like a whole person instead of a number.

As an IVF team we should all remember our patients are real people with real feelings and real emotions and we should all strive every day to fulfill these goals and to help each couple achieve a pregnancy and experience the joys of parenthood.

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As an IVF team we should all remember our patients are real people with real feelings and real emotions.
Assessing sHLA-G secretion: a useful marker for embryo selection on day 3

by Levent Keskinetpe, PhD, HCLD from the Sher Institute in Las Vegas

Human embryos for transfer are conventionally selected based on the evaluation of the morphologic criteria. Identifying the ‘best’ embryos for transfer has been a clinical challenge for more than 2 decades. Morphological assessment of embryos prior to transfer and prolonged embryo culture for 5 or 6 days post-fertilization have improved implantation potential. However, while morphological evaluations furnish clues that can enhance proficiency at choosing the best pre-embryos for transfer, such morphologic analyses have an inherently limited ability to predict the implantation potential of individual embryos.

The ultimate goal of ART is to achieve singleton pregnancies.

The ultimate goal of ART is to achieve singleton pregnancies. Because the number of embryos transferred is closely related to success rate, it is challenging to decide how many embryos should be transferred. It would be more convenient if an equally effective selection could be performed at an earlier stage. In an attempt to determine embryo implantation competency on day 3, we examined prospectively the soluble HLA-G (sHLA-G) secretion in the media surrounding of embryos at 44-46 h after ICSI.

Human leukocyte antigen-G (HLA-G), a MHC class I gene, is of particular interest in reproductive biology because of its specific expression on cytotrophoblast cells, its role in protecting of the developing conceptus from destruction by the maternal immune response and its presence in the supernatants surrounding early embryos in culture.

Samples of media surrounding a single embryo was collected in a 0.5 ml of Eppendorf tube, immediately frozen, stored. These samples were subsequently thawed and a specific ELISA test was run for sHLA-G expression. Grading for sHLA-G expression was established as follows: “Negative” (mean OD =< 0.190 ± 0.056), “positive” and (mean OD =>0.190 ± 0.056).

Thirty eight (38) out of the 51 patients (74 %) who had all embryos tested “positive” for sHLA-G expression, transferred, achieved clinical pregnancies with at least one viable (ultrasound confirmed) implantation. The implantation rate was 44% (51/117). Fifty six patients who had all transferred embryos tested “negative” for sHLA-G expression had 23% (13/56) pregnancy rate and the implantation rate was 14% (20/146).

This data presents a possible opportunity, for the first time, to select those embryos with the highest implantation potential and competency, for transfer. We believe that the data presented supports the potential of measuring the embryos own genetic expression of “competency”, evidenced by its ability to produce sHLA-G 46 hours post-ICSI, as a measure of its implantation potential. It also establishes a rational basis for transferring no more than two embryos at a time, setting the stage for the potential elimination of high order multiple pregnancies following IVF.

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Success With Blastocyst Stage Transfer Is Not Limited By Maternal Age When Taking Into Account The Number And Quality Of Blastocysts Transferred

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ART Program of Alabama, Birmingham, AL
Poster presentation (P-220), 2004 ASRM meeting, Philadelphia, PA

Introduction: Traditionally, when undergoing In Vitro Fertilization (IVF) procedures, embryos have been transferred into the uterus at the 4-8 cell developmental stage. This usually occurred two to three days following oocyte retrieval. Advances in culture media and culture conditions have led to an improvement in embryo growth to the blastocyst stage and subsequently an increase in the number of blastocyst stage transfers during IVF. The blastocyst is more representative of the embryonic genome as compared to the 4-8 cell embryo. Extending the culture time for embryo transfer from 3 to 5 days may favor a natural selection against certain chromosomal abnormalities. Typically, fewer blastocysts are needed for embryo transfer (ET) to yield an equivalent or higher chance of pregnancy as compared to the 4-8 cell embryo, hopefully reducing the chance of a high-order multiple gestation. Furthermore, the presence of a high quality blastocyst at ET may reduce the chance for a twin gestation through single blastocyst transfer. The impact of maternal age on this system needs further definition.

Objectives:
The objectives of this study were 1) to compare the effect of maternal age on the implantation, ongoing/delivered and multiple gestation rates during IVF with blastocyst transfer and 2) to compare the effect of the number and quality of blastocysts transferred on the implantation, ongoing/delivered pregnancy and multiple gestation rates during IVF with blastocyst transfer.

Design:
This study was designed to retrospectively review the outcome of all cycles with blastocyst transfer during a three and one-half year period. Outcome was evaluated by implantation rate (IR), defined as the number of ongoing fetal heartbeats and/or live births per embryo transferred, ongoing/delivery rate (ODR), defined as the number of ongoing (more than 12 weeks) pregnancies and/or deliveries per transfer, multiple gestation rate (MGR), defined as the number of ongoing and/or delivered pregnancies with more than one fetal heart beat and/or live birth and high-order multiple gestation rate (HOMGR), defined as the number of ongoing and/or delivered pregnancies with more than two fetal heart beats and/or live births. The number of monozygotic twins per ongoing/delivered pregnancy (MZTR) was also reviewed. The effect of the number and quality of blastocysts transferred on the above rates was also considered in the data analysis.

Materials and Methods:
The data consisted of 194 blastocyst transfer cycles during the period January 2001 through June 2004. During 2001-2003, cycles in which there were 6 or more embryos with 6 or more cells and ≤15% fragmentation available on day 3 of embryo culture were scheduled for blastocyst transfer. After this period, the criteria for blastocyst transfer was extended to those cycles in which 2 embryos of good quality in excess of the number to be transferred on day 3 of embryo culture were present. Occasionally, other criteria for blastocyst transfer included: failed IVF with day 2-3 transfer, sub-optimal endometrium on day 2-3 of embryo culture and patient desire. All cycles were included in the study and were grouped according to maternal age, based on oocyte source, and number and quality of blastocysts transferred. Controlled ovarian hyperstimulation routinely included three to five amps of follicle stimulating hormone with one base amp of luteinizing hormone daily. Ideally, human chorionic gonadotrophin was given when 2 or more follicles were 1.8 mm and E2 >1000 pg/mL. Embryos were cultured in microdroplets under oil. Various culture media were utilized during this time. Currently, Global media (LifeGlobal, IVFonline), a simple one step system, supplemented with 10% human serum albumin (LifeGlobal, IVFonline), is used for embryo culture from fertilization to blastocyst in an atmosphere of 5.5% CO₂ in air. Paraffin oil (LifeGlobal, IVFonline) is used for the oil overlay. Intracytoplasmic sperm injection was utilized in 75% of all cases. Blastocysts were transferred on day 5 of culture utilizing an echotip catheter (Cook) under ultrasound guidance. Blastocysts were graded based on the system used by Gardner, et. al.: BL1 (early), BL2 (blastocyst), BL3 (full), BL4 (expanded), BL5 (hatching) and BL6 (hatched). Fisher’s exact test and Student’s t-test were used for statistical analysis as indicated. Statistical significance was defined as P <0.05.

Continued on Page 14
**Results**

### Table 1. Treatment outcome as a function of age in a group of 194 cycles with blastocyst transfer.

<table>
<thead>
<tr>
<th>AGE</th>
<th>Blasto cyst transfers</th>
<th>Ongoing and/or delivered pregnancies (ODR)</th>
<th>Multiple gestations (MGR)</th>
<th>High-order multiple gestations (HOMGR)</th>
<th>Monozygotic twinning (MZTR)</th>
<th>Blastocysts transferred (average per ET)</th>
<th>Ongoing fetal heart beats and/or live births (IR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;35</td>
<td>156</td>
<td>84 (53.8)a</td>
<td>35 (41.7)</td>
<td>2 (2.4)</td>
<td>5 (6.0)</td>
<td>331 (2.1)b</td>
<td>121 (36.6)a</td>
</tr>
<tr>
<td>35-37</td>
<td>27</td>
<td>15 (55.6)</td>
<td>4 (26.7)</td>
<td>1 (6.7)</td>
<td>1 (6.7)</td>
<td>67 (2.5)a</td>
<td>20 (29.9)</td>
</tr>
<tr>
<td>&gt;37</td>
<td>11</td>
<td>2 (18.2)a</td>
<td>1 (50.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>28 (2.5)b</td>
<td>3 (10.7)a</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.045a</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001b, 0.002b</td>
<td>0.0006a</td>
</tr>
</tbody>
</table>

### Table 2. Treatment outcome as a function of the highest grade of blastocyst transferred in a group of 194 transfers.

<table>
<thead>
<tr>
<th>Highest Blastocyst Grade</th>
<th>Morula, BL1, BL2</th>
<th>BL3, BL4, BL5, BL6</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blastocyst transfers</td>
<td>48</td>
<td>146</td>
<td></td>
</tr>
<tr>
<td>Ongoing and/or delivered pregnancies (ODR)</td>
<td>14 (29.2)</td>
<td>87 (59.6)</td>
<td>0.0004</td>
</tr>
<tr>
<td>Multiple gestations (MGR)</td>
<td>4 (28.6)</td>
<td>36 (41.4)</td>
<td>NS</td>
</tr>
<tr>
<td>High-order multiple gestations (HOMGR)</td>
<td>0 (0.0)</td>
<td>3 (3.4)</td>
<td>NS</td>
</tr>
<tr>
<td>Monozygotic twinning (MZTR)</td>
<td>0 (0.0)</td>
<td>6 (6.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Blastocysts transferred (average per ET)</td>
<td>106 (2.2)</td>
<td>320 (2.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Ongoing fetal heart beats and/or live births (IR)</td>
<td>18 (17.0)</td>
<td>126 (39.4)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

### Table 3. Treatment outcome as a function of the total number of blastocysts transferred in a group of 194 transfers.

<table>
<thead>
<tr>
<th>Number of Blastocysts Transferred</th>
<th>2 or less</th>
<th>More than 2</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blastocyst transfers</td>
<td>154</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Ongoing and/or delivered pregnancies (ODR)</td>
<td>80 (51.9)</td>
<td>21 (52.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Multiple gestations (MGR)</td>
<td>29 (36.3)</td>
<td>11 (52.4)</td>
<td>NS</td>
</tr>
<tr>
<td>High-order multiple gestations (HOMGR)</td>
<td>0 (0.0)</td>
<td>3 (14.3)</td>
<td>0.02</td>
</tr>
<tr>
<td>Monozygotic twinning (MZTR)</td>
<td>6 (7.5)</td>
<td>0 (0.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Blastocysts transferred (average per ET)</td>
<td>303 (2.0)</td>
<td>123 (3.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ongoing fetal heart beats and/or live births (IR)</td>
<td>109 (36.0)</td>
<td>35 (28.5)</td>
<td>NS</td>
</tr>
</tbody>
</table>

### Table 4. Treatment outcome as a function of the highest grade of blastocyst transferred in a group of 154 transfers with two or less blastocysts transferred.

<table>
<thead>
<tr>
<th>Highest Blastocyst Grade</th>
<th>Morula, BL1, BL2</th>
<th>BL3, BL4, BL5, BL6</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blastocyst transfers</td>
<td>36</td>
<td>118</td>
<td></td>
</tr>
<tr>
<td>Ongoing and/or delivered pregnancies (ODR)</td>
<td>11 (30.6)</td>
<td>69 (58.5)</td>
<td>0.0006</td>
</tr>
<tr>
<td>Multiple gestations (MGR)</td>
<td>3 (27.3)</td>
<td>26 (37.7)</td>
<td>NS</td>
</tr>
<tr>
<td>High-order multiple gestations (HOMGR)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Monozygotic twinning (MZTR)</td>
<td>0 (0.0)</td>
<td>6 (8.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Blastocysts transferred (average per ET)</td>
<td>69 (1.9)</td>
<td>234 (2.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Ongoing fetal heart beats and/or live births (IR)</td>
<td>14 (20.3)</td>
<td>95 (40.6)</td>
<td>0.002</td>
</tr>
</tbody>
</table>
Table 5. Treatment outcome as a function of the highest grade of blastocyst transferred in a group of 40 transfers with more than two blastocysts transferred.

<table>
<thead>
<tr>
<th>Highest Blastocyst Grade</th>
<th>Morula, BL1, BL2</th>
<th>BL3, BL4, BL5, BL6</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blastocyst transfers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ongoing and/or delivered pregnancies (ODR)</td>
<td>3 (25.0)</td>
<td>18 (64.3)</td>
<td>0.05</td>
</tr>
<tr>
<td>Multiple gestations (MGR)</td>
<td>1 (33.3)</td>
<td>10 (55.6)</td>
<td>NS</td>
</tr>
<tr>
<td>High-order multiple gestations (HOMGR)</td>
<td>0 (0.0)</td>
<td>3 (16.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Monozygotic twinning (MZTR)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Blastocysts transferred (average per ET)</td>
<td>37 (3.1)</td>
<td>86 (3.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Ongoing fetal heart beats and/or live births (IR)</td>
<td>4 (10.8)</td>
<td>31 (36.0)</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Results and Discussion:
An acceptable ODR and IR were achieved with a blastocyst stage transfer up to age 37 (Table 1). The ODR and IR were significantly lower for age >37, however the data are based only on 11 total ETs in this group. The two pregnancies in patients’ age >37 occurred in a group of 7 ETs with more than two blastocysts transferred (2/7, 28.6%). When a BL3 grade blastocyst or better was the highest-grade blastocyst transferred, a significantly higher ODR and IR were observed (Table 2). Also higher were MGR, HOMGR and MZTR in this group. All three high-order multiple gestations in the data were triplets as a result of more than two blastocysts transferred. Two patients received three blastocysts due to previous cycle history and elevated FSH; one patient received four blastocysts due to severe male factor (all were age <38). There was no advantage for ODR and IR when transferring more than 2 blastocysts (Table 3), with possibly the exception of age >37. There was a disadvantage to transferring more than two blastocysts when blastocyst quality was BL3 or better, in that HOMGR was significantly increased (Table 4 v. 5; 0.0% v. 16.7%, p<0.02, not shown). The MGR and the HOMGR were not increased when transferring more than 2 lower quality blastocysts (morula – BL2). MZT occurred seven times in the 194 transfers (3.6%): two patients selectively reduced (one miscarried and one delivered a singleton), three patients spontaneously reduced (two delivered non-monozygotic twins, one is ongoing with non-monozygotic twins), one delivered monozygotic twins and one is ongoing with monozygotic twins. Interestingly, all MZT occurred when only two blastocysts, at least one of which was expanded - hatching (BL4-5), were transferred. Of note, all patients, regardless of age, undergoing extended culture for blastocyst transfer based on the criteria presented, received an ET.

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Patent #6013119, #6200362, #6225110
Conclusion:
1. Women age 35-37 have similar ODR and IR as women <35 with only a slight, although significant, difference in the number of blastocysts transferred. Therefore, no more than two blastocysts should be transferred in women up to 37 years old for a suitable ODR and IR.
2. Women >37 years old had a significantly lower ODR and IR with an equivalent number of blastocysts transferred as compared to women 35-37 years old. Therefore, more than two blastocysts may need to be transferred for a satisfactory ODR and IR in women >37 years old.
3. A full to hatched blastocyst (BL3-6) has more than twice the IR as compared to an earlier blastocyst, leading also to a higher potential for HOMGR when transferring more than two of these blastocysts.
4. MZT occurs equally among age groups up to 37. The small number of transfers in women >37 years old may not allow for observation in this group. MZT occurs more frequently in full to hatched blastocysts as compared to earlier blastocyst stages.
5. Increasing the number of blastocysts transferred to more than two, especially in women up to 37 years old, significantly increases the HOMGR, and potentially the MGR, without increasing the overall ODR and IR.
6. The presence of a full to hatched blastocyst (BL3-6) for women up to 37 years old at the time of transfer may provide the means to eliminate, with the exception of MZT, multiple gestations without decreasing the ODR through the transfer of a single blastocyst.

References:

You can contact Debbie Merryman at: dcmerryman@aol.com
Although ES cells are usually derived from culture of the inner cell mass of the preimplantation blastocyst, as shown in mice and in humans, the possibility of the establishment of ES cells from the morula in mink, and cow, and also from embryonic germ cells have also been reported. The current NIH repository of ES cells contains 78 lines, of which only 11 have met NIH scientific criteria (see NIH website), including the presence of L-alkaline phosphatase (TRA-2-39), Oct-4, telomerase, high molecular weight glycoproteins (antibodies TRA-1-60, TRA-1-81), stage specific embryonic antigens (SSEA-3, SSEA-4), euploid karyotype and teratoma formation in SCID mice.

We developed an original technique for the establishment of ES cell lines from human embryos at the morula stage, which were shown to meet all the above NIH criteria mentioned. To establish human ES cell lines from morulae, the zona pellucida is removed and the morula placed under a middle density feeder layer. Within several days, cells outgrow and spread into the feeder layer. The primary cell disaggregation is performed with EDTA or EGTA, and the loose cells are transferred back to the feeder layer to proliferate. Fast proliferating colonies are isolated and propagated further.

No morphological differences between human ES-cells originating from ICM and from morula were observed, except for being more heterogeneous, as well as the pattern of above marker expression. The established human ES-cell lines are maintained in vitro from 10 to 15 passages before freezing in sufficient amounts with control thaw out. There are also no differences in the efficiency of obtaining ES cell lines depending on the source of preimplantation embryo.

To induce the differentiation of human ES cells in different cell types, the cells are cultured to form embryonic bodies, which are isolated with subsequent disaggregation and plating of clusters of cells. The cultured clusters of cells show a wide range of cell types belonging to ectoderm, endoderm and mesoderm, and spontaneously differentiate in vitro into a variety of cell types, including neuron-like cells with dendrites and contracting primitive cardiocyte-like cells. Controlling differentiation into pure populations of specific neural cells may eventually form the basis of therapy for some neurodegenerative disorders and spinal injuries. These developments provide an obvious potential for the therapeutic use of the embryonic stem cells in clinical practice.

The above developments have made it possible to initiate the establishment of a repository of ES cell lines with different genetic abnormalities. Although initially the major goal of the establishment of human ES cell lines was the development of the cell-replacement therapies, it is presently obvious that human ES cell lines will have an importance role in the studies of mechanisms of genetic disorders through generating the sources of normal and genetically abnormal cells and tissues.

The establishment of ES cell lines with genetic disorders has become possible with introduction of PGD, so that the mutation free embryos are transferred while those affected provide a valuable source of ES cell lines with genetic abnormalities, using the above techniques for the initiation of ES cells lines from preimplantation embryos. This also provides a unique opportunity to investigate the potential of establishing ESC lines depending on the genotype.

Based on our ongoing PGD work described elsewhere (Verlinsky & Kuliev, 2000), we attempted to establish ES cell lines from embryos with a variety of single gene and chromosomal disorders. PGD is an important source for the ES cell lines, because the embryos obtained from PGD are well tested, with the genotype of the potential ES cell line known from the onset. Our current experience shows that the efficiency of the derivation of ES cell lines from the embryos with single gene disorders is not affected. This is demonstrated by the establishment of the first ES cell line repository with different genetic abnormalities, which presently contains more than 50 human ES cell lines. This repository contains human ES cell lines obtained from embryos with single gene disorders, such as thalassemia (HBB), neurofibromatosis type 1 (NF1), Marfan syndrome (FBN1), myotonic dystrophy (DMPK), Becker muscular dystrophy (BMD), fragile-X syndrome (FMR1), and Huntington disease (HD).

**FURTHER READING**

You can contact Yury Verlinsky, PhD and Anver Kuliev, MD, PhD at: mail@reproductivegenetics.com
between 1975 and 2003, 4,010 patients have undergone meticulously accurate microscopic vasectomy reversal by one surgeon at one institution. A total of 3,904 of those cases had available records, and 1,735 were successfully contacted to obtain reliable long term data. Of these, 1,556 (89.7%) were able to establish a pregnancy in their female partner resulting in a total of 2,111 pregnancies.

When there was no sperm in the vas fluid at the time of vasectomy reversal, vasoepididymostomy was performed rather than vasovasostomy. 1,581 patients underwent bilateral vasovasostomy, 1,184 underwent vasoepididymostomy on one side and vasovasostomy on the other side, and 1,139 underwent bilateral vasoepididymostomy.

There were no sperm in the post-operative ejaculate of 2.1% of patients after vasovasostomy, and in 10.3% of patients after bilateral vasoepididymostomy. Thus, on average, a patency rate of 96.2% in the total group of patients was achieved. When the vasectomy was less than 10 years prior to reversal, the patency rate was 98%. When the vasectomy was 10 or more years prior to reversal, the patency rate was 93%. Post-operative sperm counts were greater than 5 million per cc in 77.7% of patients.

Pregnancy was eventually achieved in the partners of 92.5% of patients undergoing bilateral vasovasostomy, and in the partners of 84.3% of those undergoing bilateral vasoepididymostomy. The pregnancy rate did not differ with patients that had a sperm count of greater than 5 million sperm per cc. The pregnancy rate for less than 5 million sperm per cc was 78.3% and when there were greater than 5 million sperm per cc the pregnancy rate was 91.9%. Sperm count itself is not critical to pregnancy rate. There is a wide variation in sperm count among normal fertile men, and thus there is a wide variation in semen analysis after vasectomy reversal. However, as long as there was no partial obstruction (as would be indicated by discrepancy between sperm count and quantitative testis biopsy), the long-term pregnancy rate in this population approximates previously reported population norms.

Although the duration of time between vasectomy and vasectomy reversal had an impact on pregnancy rate, the greatest impact was the age of the wife; 94.2% of wives under age 30 at the time of the vasectomy reversal established a pregnancy, but only 61.1% of wives age 40 or older established a pregnancy.

We conclude that microsurgical vasectomy reversal is preferable to sperm retrieval and ICSI, since the pregnancy rate appears to be higher with this technique than with sperm retrieval and ICSI. It does not appear that sperm antibodies, or testicular damage are likely to account for failure to achieve pregnancy after vasectomy reversal. Rather it is likely to be related to partial or complete obstruction following surgery, or to the fertility of the female partner.

You can contact Sherman J. Silber, MD at: silber@infertile.com
THE CLINICAL APPLICATION OF METAPHASE I (MI) HUMAN OOCYTES OBTAINED FROM STIMULATED CYCLES

by Hanna Balakier, PhD

In gonadotropin-stimulated cycles, some of the collected oocytes are immature, either at the germinal vesicle stage (GV) or at metaphase I of the first meiotic division. Unlike GV oocytes that require a prolonged incubation period (minimum 8-10 hours), metaphase I oocytes frequently mature within a few hours of in-vitro culture. During this time they extrude the first polar body (1PB) and reaching metaphase II phase of the second meiotic division (MII). At this stage they may be suitable for sperm injection (ICSI) at the same time as their sibling in vivo matured MII oocytes. In daily practice these oocytes are usually discarded due to the increased possibility of their abnormal embryonic development. However, in the case of poor responders or in patients where the presence of immature oocytes prevail, the use of MI oocytes for in vitro fertilization may play an important role in increasing the number of embryos available for transfer and thus, enhancing the chances of pregnancy.

Recent studies on human MI rescued oocytes from stimulated cycles indicated that the process of their maturation is not fully complete upon reaching the MII stage. It was shown that final maturation of MII-arrested oocytes is crucial for their capacity to undergo normal activation and cleavage. Oocytes injected soon after extrusion of the 1PB remain unfertilized but with prolonged time of MII arrest before ICSI (1, 2 and 3-6 hours), the proportion of fertilized, normal zygotes containing two pronuclei and two polar bodies gradually increases to 25%, 43% and 61% respectively. This may suggest that a minimum of 3 hours of MII arrest is required to obtain reasonable fertilization rates comparable with those recorded for the in-vivo matured siblings (77%).

The developmental capacity of human embryos originating from MI oocytes is significantly reduced, and a high proportion of embryos are arrested soon after the first two divisions. It was also noted that embryos originating from in-vitro matured MI oocytes exhibited multinucleated blastomeres (MNB) in nearly double frequency when compared with embryos from in-vivo matured MII oocytes. Interestingly this nuclear abnormality suggesting the existence of chromosomal aberrations is especially manifested in the embryos when ICSI is performed shortly after 1PB extrusion (36% versus 23% when ICSI performed at 1-1.5 hours, and 3-6 hours after extrusion of 1PB). Based on recent animal and human studies, it is now clear that some profound changes take place during the early phase of MII arrest, which are extremely important for assembly, and normal function of the meiotic spindle and thus the chromosomal status of growing embryos. Therefore, it appears that defining optimal incubation periods for oocyte maturation as well as timing of their ICSI are the key factors for the acquisition of oocyte developmental competence.

Taking into consideration the fact that clinical application of in-vitro matured MI oocytes from stimulated cycles still remains questionable; caution should be exercised when they are used in assisted reproductive technology programs (minimum 4-6 hours of culture after PB extrusion, observation of MNB). Hopefully in the near future, the improvement of culture conditions and a defined optimal time for oocyte maturation as well as ICSI may enhance the results of the various human in vitro maturation systems.

For more details see: Human Reproduction Vol. 19, No. 4, pp 982-987, 2004

You can contact Hanna Balakier, PhD at: hanna@createivf.com
Bovine Oocytes and Embryos as Models for Human ART

by Don Rieger, PhD

New and improved techniques for assisted reproduction in humans must be tested for efficacy and safety before they can be introduced into clinical practice. For practical, ethical, and legal reasons, basic experiments can not be done on human gametes or embryos, and consequently the gametes and embryos of other species must be used as models. The mouse embryo has been extensively used as a general model for early development and is routinely used for quality control testing of human ART media and materials. It is widely available, relatively inexpensive, and there is a wealth of information about its developmental genetics, morphology, and physiology. However, many aspects of the developmental biology of the mouse embryo differ significantly from those of the human embryo and consequently embryos of other species are, in many cases, better models.

Clearly, non-human primates (NHP) are phylogenetically closest to humans and would therefore be the best models for human embryo development (Bavister 2004). Unfortunately, as noted by Bavister, most of the studies of non-human primate ART have been done in the rhesus macaque, but there is now an intense demand for these animals for AIDS research. Moreover, human ART has progressed much more quickly than that for non-human primates. For these and other reasons, it is doubtful that non-human primates will be practical as a model for human ART in the immediate future.

For both biological and practical reasons, bovine oocytes and embryos are very useful models for human ART. In a recent review, Menezo and Herubel (2002) have argued that the bovine embryo is a better model than the mouse embryo for studies of oocyte maturation, fertilization, extended culture, and cryopreservation. As well, the development and application of assisted reproductive technologies in cattle have usually preceded, and been the basis for, their practical application in humans. Examples are embryo transfer, sperm and embryo cryopreservation, embryo biopsy for cytogenetic evaluation, embryo culture, and in-vitro oocyte maturation (IVM) (Betteridge and Rieger 1993). These techniques are widely used in the field (sometimes literally). According to a survey by the International Embryo Transfer Society, more than 500,000 in-vivo developed cattle embryos, and more than 83,000 IVF cattle embryos were transferred in 2002, world-wide (Thibier 2003). Studies of bovine IVM and extended embryo culture are two areas of particular significance to human ART.

Edwards (1965) observed that oocytes from a variety of species will undergo spontaneous nuclear maturation when removed from the follicle. However, many subsequent studies showed that the in-vitro fertilization and development of such oocytes is very limited, and this was attributed to the lack of cytoplasmic maturation. Consequently, human IVF has relied on the collection of in-vivo matured oocytes, collected after the LH surge. The first successful IVF in cattle similarly relied on in-vivo oocyte maturation (Brackett et al. 1982), but the extensive hormone monitoring required for this approach is impractical for routine application in cattle. A great many studies, notably by Leibfried-Rutledge and others in Neal First’s laboratory in Madison, Wisconsin, led to the birth of the first calf from an in-vitro matured oocyte (Leibfried-Rutledge et al. 1986). Based largely on these studies, Trounson et al. (1994) successfully matured human oocytes taken from small follicles of PCOS patients. For the most part, serum and pharmacological concentrations of gonadotrophins are used in bovine IVM, but many studies have shown that physiological concentrations of EGF, IGF-1 and other growth factors are also effective (Smits et al. 2001). It seems likely that the use of growth factors will ultimately supplant the use of serum and gonadotrophins in bovine and human IVM.

Extended culture of human embryos for transplant at the blastocyst stage has been suggested as an approach to better evaluation of developmental potential. Fewer embryos (ideally one) can be transferred and yield good pregnancy rates with a much reduced chance of multiples (Gardner and Lane 2003). With very few exceptions, bovine IVF embryos are cultured to the morula or blastocyst stage because, unlike in the human, cleavage stage bovine embryos will not survive in the uterus. Initially, this problem was circumvented by temporary culture in the ligated sheep oviduct, and later in co-culture with oviductal or other cells. Except in special situations, these techniques have been replaced by culture in defined culture media, containing serum albumin, but no somatic cells or serum (Thompson 2000). In one example (Gandhi et al. 2000), approximately 25% of the original immature bovine oocytes developed to the blastocyst stage following IVM and culture in defined media.

In cattle, transfer of one embryo is the norm, because of the risk of freemartinism. As for human embryos, morphology is used to predict the viability of bovine embryos. Although this works on a population basis, it is notoriously unreliable for an individual embryo, and efforts have been made to predict viability based on metabolism for more than 25 years (Renard et al. 1978; Rieger 1984). Recently, highly sensitive probes have been used to measure oxygen uptake by individual bovine...
embryos (Shiku et al. 2001; Lopes et al. 2004) and could be applied to human embryos.

The bovine embryo is also an important model for the possible negative effects of embryo culture, originating from the observations in two landmark papers. In the first, Iannaccone (1984) showed that mouse blastocysts survived exposure to low concentrations of methylnitrosourea, but the mortality rate of the pups originating from these blastocysts was significantly increased in the first year following birth. In the second, Willadsen et al. (1991) showed that there was a significant incidence of high birth weight, birth defects and perinatal death in calves produced by cloning of early bovine embryos. This was later dubbed the large offspring syndrome (LOS) and was shown to occur in cattle and sheep fetuses produced by IVM/IVF as well as by cloning (Kruip and den Daas 1997). Together, these observations indicated that the environment to which an early embryo is exposed can have significant and deleterious effects on development at a much later stage. In humans, low birth weight of IVF babies, even among singletons, is the usual concern, but DeBaun et al. (2003) have suggested that Beckwith-Wiedemann syndrome (BWS) may be related to in-vitro culture. It has yet to be determined whether these LOS and BWS are truly analogous, but understanding LOS has enormous implications for human developmental biology.

Several recent developments will facilitate the study of the developmental biology of the bovine embryo, and its use as a model for the human embryo. A $53 million (US) project to sequence the bovine genome is being undertaken through a collaboration of U.S., Australian, New Zealand and Canadian researchers, and a microarray of over 23,000 bovine transcripts is commercially available. It has been shown that mRNA expression differs between in-vivo and in-vitro produced bovine embryos (Wrenzycyki et al. 2001), and complete sequencing of the bovine genome and the availability of microarrays will undoubtedly reveal further differences. As well, bovine oocytes and early embryos up to the blastocyst stage are now commercially available, making this model available to many more research laboratories.

References


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COMPARISON OF A SINGLE MEDIUM WITH SEQUENTIAL MEDIA FOR DEVELOPMENT OF HUMAN EMBRYOS TO THE BLASTOCYST STAGE

Melanie R. Freeman\(^1\) and Don Rieger\(^2\)

\(^1\)Nashville Fertility Center, Nashville, TN, USA and \(^2\)LifeGlobal, Guelph, ON, Canada

<table>
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<th>Day</th>
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<th>Cleavage Grade</th>
<th>Blastoscyt Grade</th>
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<tr>
<td>5</td>
<td></td>
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<td></td>
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<tr>
<td>6</td>
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</table>

Mean Scores

Presented at IFFS 2004 Meeting
ABSTRACT

Introduction

It has been suggested that culture of human embryos to the blastocyst stage requires the sequential use of two media for culture to Day 3, and a second medium for culture thereafter. However, it has been shown that human embryos can be successfully cultured in the same medium throughout. At the Nashville Fertility Center, embryos are routinely cultured in IVF-One (InViroCare) from Day 1 to Day 5, and then in G2 (Gardner & Lana, Hum Reprod Update, 3, 367-382, 1997). From Day 3 to Day 5 or Day 6. The objective of this experiment was to directly compare the development of embryos in this sequential media system (IVF-One/G2) with development in a single medium (Global, LineGlobal) from Day 1 to Day 5, and from Day 3 to Day 5 or Day 6.

Materials and Methods

On day 3 embryos were retrieved from 37 women (mean age = 32.1; range 23-39) undergoing IVF treatment between December, 2003 and March, 2003. Following fertilization in Ovocyte's Advantage medium (Cooper Surgical), the eggs were cultured in equal groups. The embryos of the Global group (N = 210) were cultured in IVF-One medium until Day 3, and then in G2 medium until Day 5 or Day 6. Embryo development and morphology were evaluated on Days 2, 3, 5, and 6, according to the criteria shown in Table 1. Appropriate development was defined as being >5 cells on Day 2, >7 cells on Day 3, and expanded blastocyst on Day 5 or Day 6. After evaluation on each day, embryos were removed from the treatment groups if they were degenerate or retracted, or to be transferred or frozen (Table 2).

For the statistical analyses, the scores for embryonic development, morphology, and blastocyst expansion on each day were compared between treatment groups using Kruskal-Wallis tests. The proportion of embryos with appropriate development on each day was compared between treatment groups by Chi-square analysis.

Table 1. Grades and Scores for Embryo Development and Morphology

<table>
<thead>
<tr>
<th>Measure</th>
<th>Grade</th>
<th>Description</th>
<th>Numerical Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryo Development</td>
<td>Degenerate</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1+6 Cell</td>
<td></td>
<td>1+6</td>
</tr>
<tr>
<td></td>
<td>Morula</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Blastocyst</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Cleavage Stage Morphology</td>
<td>A</td>
<td>Symmetrical blastomere, no fragmentation</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>Unsymmetrical blastomere, or &lt;10% fragmentation</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>10-22% fragmentation</td>
<td>1</td>
</tr>
<tr>
<td>Blastocyst Morphology</td>
<td>A</td>
<td>Inner Cell Mass</td>
<td>Trophectoderm</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>Tightly packed, many cells</td>
<td>Many cells forming a cohesive epithelium</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>Loosely grouped, few cells forming a loose epithelium</td>
<td>Very few large cells</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>Very few cells</td>
<td>Abnormal</td>
</tr>
<tr>
<td>Blastocyst Expansion</td>
<td>A</td>
<td>Early blastocyst with a blastocoele just appearing</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>A distinct single cavity equal to one-quarter to one-half of the volume of the embryo</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>A full blastocyst with a blastocoele completely filling the embryo</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>Fully expanded with a shrinking zona, and a distinct ICM and trophoderm layer</td>
<td>4</td>
</tr>
</tbody>
</table>

Results

The results are displayed in Table 2. The top four rows show the results for each day. The columns show the measures of development, morphology, blastocyst expansion and appropriate development. The bottom row shows the mean scores for the measures for all days. The results for the Global group are shown in black (■), and the results for the IVF-One/G2 group in gray (■).

Development score was greater in the Global than in the IVF-One/G2 group on Day 2 and on Day 5 but was not different between the Global and IVF-One/G2 groups on Day 5 or Day 6.

The morphological quality of cleavage stage embryos and blastocysts was not different between treatment groups on any of Days 2, 3, 5 or 6.

The proportion of embryos having appropriate development was greater in the Global group than in the IVF-One/G2 group on Days 3 and 5, but not on Days 2 or Day 6.

Table 2. Number and Disposition of Embryos on Each Day

<table>
<thead>
<tr>
<th>Medium</th>
<th>Global</th>
<th>IVF-One/G2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 2</td>
<td>213</td>
<td>210</td>
<td>423</td>
</tr>
<tr>
<td>Removed</td>
<td>Degenerate</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Day 3</td>
<td>192</td>
<td>210</td>
<td>402</td>
</tr>
<tr>
<td>Removed</td>
<td>Degenerate</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>i-cell</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Transferred</td>
<td>10</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>Day 5</td>
<td>191</td>
<td>199</td>
<td>390</td>
</tr>
<tr>
<td>Removed</td>
<td>Degenerate</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Transferred</td>
<td>42</td>
<td>34</td>
<td>76</td>
</tr>
<tr>
<td>Frozen</td>
<td>24</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>Day 6</td>
<td>137</td>
<td>140</td>
<td>277</td>
</tr>
<tr>
<td>Frozen</td>
<td>26</td>
<td>37</td>
<td>63</td>
</tr>
</tbody>
</table>

Discussion and Conclusions

Development, morphological quality, and blastocyst expansion of human embryos cultured in a single medium (Global) were as good, or better, than those for embryos cultured in a sequential media system (IVF-One/G2).

These observations agree with the report of Nguyen and Renczisly (Reprod. Biomed. Online, 3, 133-140, 2003) who showed that blastocysts obtained using the same media were not different between human embryos cultured in K200 (AA) from Day 1 to Day 5, compared with embryos cultured in a sequential media system.

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