Preliminary clinical experience with human blastocyst development in vitro without co-culture

B.Behr, T.B.Pool, A.A.Milki, D.Moore, J.Gebhardt and D.Dasig

1Department GYN/OB Stanford University Medical Center, Stanford, CA 94305 and 2Fertility Center of San Antonio, San Antonio, USA

3To whom correspondence should be addressed

This preliminary analysis was designed to quantify blastocyst development of supernumerary embryos without the use of feeder cells, conditioned medium or whole serum. Embryos derived from in-vitro fertilization (IVF) that were not transferred or cryopreserved were included in this study. Ova were harvested for IVF after a standard ovarian stimulation with gonadotrophin-releasing hormone agonist/human menopausal gonadotrophin (GnRHa/HMG) or follicle-stimulating hormone (FSH). Ova were collected and cultured in 150 µl droplets of P1 medium under mineral oil, in groups at 37°C under 5% CO2, 5% O2, 90% N2 (group A) or under 5% CO2 in air (group B) environment. Embryo transfer was performed 72 h post-harvest. Viable embryos not transferred or cryopreserved were placed in blastocyst medium and cultured for an additional 48 h in 5% CO2 in air. Embryos that exhibited an expanded blastocoelic cavity and well-defined inner cell mass at 120 h were counted. Of 838 supernumerary embryos cultured, 448 (53.5%) reached the expanded blastocyst stage by 120 h of culture. Patients were given the option of cryopreservation at that time. The embryos were cryopreserved using a standard protocol with serial addition of glycerol. Embryos reaching the blastocyst stage after more than 120 h of culture were not included. There was no difference in the proportions of blastocyst development between group A, 217/410 (53.5%) and group B, 231/428 (54%). To date, 16 patients have each had up to three thawed blastocystos transferred, out of whom seven became pregnant. This report demonstrates that a simple system of sequential culture generated acceptable, viable blastocyst development (54%) with supernumerary embryos, without the use of feeder cells, conditioned medium or whole serum. Recognizing the differential metabolic requirements of early and late cleavage stage embryos has enabled the application of a glucose/phosphate-free simple culture medium (P1) for up to 72 h of culture and a complex, glucose-containing medium (blastocyst medium) for subsequent blastocyst development.

Key words: blastocyst/development/embryo culture/implantation/sequential culture

Introduction

The potential advantages of the uterine transfer of blastocysts following human in-vitro fertilization (IVF) have been enumerated recently by Gardner and Lane (1997) and include synchronization of the embryo with the female reproductive tract with increasing implantation rates, thereby reducing the need for the transfer of multiple embryos. The drawbacks to implementing this strategy universally with conventional culture technology, however, have been twofold: (i) the inability to produce blastocysts both routinely and efficiently and (ii) the low viability realized upon transfer of these blastocysts. Low production and accompanying low viability of blastocysts during human IVF as a manifestation of the inability of any single culture medium to meet the changing nutritional requirements of preimplantation embryos were proposed initially by Gardner (1994). He instead suggested using different media during embryo culture, each optimized to meet the nutritional requirements specific to evolving embryonic stages. To this end, Gardner and his colleagues have developed a sequential culture system employing two media, G1 and G2. The design of these was accomplished largely through two experimental approaches. First, the concentrations of glucose, lactate and pyruvate in G1 and G2 were derived from measurements made of the concentrations of these metabolites in human reproductive tract fluid, both tubal and uterine, at various times during the menstrual cycle (Gardner et al., 1996). Secondly, the optimization of the amino acid composition for each medium was performed through exhaustive experimentation in a mouse model system and has been summarized succinctly by Lane and Gardner (1997). The ability to produce a viable human blastocyst with this sequential approach was originally demonstrated by Barnes et al. (1995) in a case report of pregnancy following in-vitro maturation of oocytes, intracytoplasmic sperm injection and subsequent blastocyst transfer. More recently, Gardner et al. (1998) reported five pregnancies resulting from eight transfers of blastocysts with a 45.5% implantation rate. This trial, though limited in scope, shows that viable human blastocysts can be produced without the need either for co-culture or whole serum. Although it is clear that sequential culture offers great hope for routine blastocyst production in clinical IVF, the exact requisites for each culture interval, and thus each medium, have not been established directly. In another recent study of human blastocyst production, IVF-50 medium (Scandinavian IVF Science AB, Gotenburg, Sweden) was used in the first culture interval and G2 was used in the second (Jones et al., 1998). Also, the embryos were regrouped by developmental stage for culture in the second interval and the G2 medium was replaced on
day 5. Some embryos were cultured for an additional day or two prior to replacement and all blastocysts were transferred after complete removal of the zona pellucida using pronase. With these modifications, a 38% pregnancy rate per transfer was realized.

Our approach to blastocyst production follows the sequential strategy, but employs a protein-supplemented, glucose/phosphate-free variant of human tubal fluid medium (‘P1 medium’; Pool et al., 1998), containing the single amino acid taurine for the first culture interval. A modified Ham’s F-10 (‘blastocyst medium’; Behr, 1997) was used for the second culture period. Both media are commercially available, are supplemented with synthetic serum substitute (SSS) and blastocysts are transferred without zona removal. In this report, we show that viable human blastocysts can be produced efficiently with P1/blastocyst medium used sequentially, suggesting that neither glucose nor a complex array of amino acids is required by early cleavage-stage preimplantation embryos during the first culture period.

The production of viable blastocysts using a simple, commercially-available sequential culture system represents an important goal in the advancement of clinical IVF. The utility of the system described in this report is still not fully known since the rate of blastocyst production was scored only on day 5 and largely by culturing supernumerary embryos after embryo transfer of the best grade embryos on day 3. However, there appears to be no glucose or inorganic phosphate requirement in the first interval. Further, the inclusion of taurine as the only amino acid in the first interval is consistent with the production of viable blastocysts as demonstrated by implantation following embryo transfer.

Materials and methods

Ova were harvested using transvaginal ultrasound guidance from patients undergoing IVF with a standard ovarian stimulation with gonadotrophin-releasing hormone agonist/human menopausal gonadotrophin (GnRHa/HMG) or follicle-stimulating hormone (FSH) at two IVF centres, groups A and B. Each couple signed the appropriate informed consent forms. The ova were collected and cultured in 150 µl droplets of P1 (Irvine Scientific, Santa Ana, CA, USA) under mineral oil, in groups of 3–5, at 37°C under a 5% CO₂, 5%O₂ and 90%N₂ environment (group A). Alternatively, the ova were collected and then inseminated in 1 ml of P1 in tubes, and transferred after fertilization into droplets of the same medium under oil in 5% CO₂ in air (group B). After verification of fertilization, all the zygotes were pooled and cultured together in one 150 µl drop. Embryo transfer was performed 72 h post harvest. Embryos that exhibited the best morphology and highest cell number were selected for embryo transfer. Embryos that were not transferred and were at least grade 3 or better (grades 1–5, 1 is best, 5 is worst) were cryopreserved. Viable embryos not transferred or cryopreserved, having at least five cells, were placed into blastocyst medium (Irvine Scientific) and cultured for an additional 48 h. Embryos that exhibited an expanded blastocoelic cavity and well-defined inner cell mass at 120 h were counted. Blastocysts were either cryopreserved or discarded depending on the request and consent of the respective patients. Blastocysts were cryopreserved using either Menezo’s multi-step glycerol protocol or Menezo’s two-step glycerol and sucrose protocol (Menezo et al., 1992).

Results

Of 838 supernumerary embryos cultured, 448 (53.5%) reached the blastocyst stage by 120 h post-retrieval. These embryos were contributed by 380 patients of whom 247 had blastocysts (65%). There was no difference in the blastocyst formation rate between the two gas phases used: group A 53% (217/410); group B 54% (231/428). Sixteen patients have had two to three thawed blastocysts transferred and clinical pregnancies were recorded in seven of those patients. The implantation rate per embryo was 19%.

Discussion

We have shown that 54% of supernumerary embryos will become blastocysts within 2 days of culture in a blastocyst medium if cultured for the first 3 days in a glucose/phosphate-free environment with the amino acid taurine (P1 medium). More importantly, by conducting both frozen and fresh embryo transfers, we have demonstrated that the blastocysts produced in this manner are viable and capable of implantation at a rate of 19% per embryo transferred. In a separate ongoing trial, 22 out of 30 patients to date have become pregnant following the transfer of up to three fresh blastocysts on day 5, with an implantation rate of 50%.

Since the first medium used in the culture sequence was completely devoid of both glucose and inorganic phosphate, neither appear to be required in the first culture interval. On the contrary, a complete lack of glucose/phosphate in culture medium for the first 3 days has been shown to improve pregnancy rates significantly when compared to a similar medium containing both (Quinn, 1995; Rawlins et al., 1997; Barrett et al., 1997; Pool et al., 1998). Furthermore, culturing embryos for 3 days in a glucose/phosphate-free environment gave significantly increased pregnancy rates over 2 days of culture prior to transfer (Carrillo et al., 1998). Conaghan et al. (1993) showed a slight enhancement of development of human embryos to the eight-cell stage by the omission of glucose compared to the inclusion of 1 mM glucose. Conaghan also demonstrated that blastocysts had more cells if glucose was omitted at the early cleavage stages. An important consideration regarding metabolic roles for glucose, other than as an energy substrate, has been raised by Gardner and Lane (1997), namely that glucose is a key entity in several crucial anabolic pathways that lead ultimately to the generation of triacylglycerols, phospholipids, mucopolysaccharides, glycoproteins and purines both for nucleic acids and the reduced oxidation-reduction co-enzyme, nicotine adenine dinucleotide phosphate.

Although the possibility exists that the low level (<0.5 mM) of glucose seen in the Fallopian tube at midcycle (Gardner et al., 1996) is required to support these anabolic needs, there are data demonstrating the existence of intracellular sources of glucose within the oocyte that could also function in these pathways. For example, when glucose and pyruvate consumption and the production of lactate were measured in single human embryos through non-invasive technology, more lactate was produced than could be accounted for by glucose uptake (Gott et al., 1990), suggesting that the remaining lactate was derived from endogenous sources, such as glycogen.
Additionally, the uptake of glucose by human embryos in medium containing 1 mM glucose is less than 10 pmol/embryo/h prior to the 16-cell stage (Hardy, 1993). To date, no studies have demonstrated the advantage of including glucose at any concentration in the first culture interval (3 days) whereas significant improvements, both to embryogenesis and pregnancy upon transfer of cleavage-stage embryos, have been reported when glucose is omitted completely. However, the inclusion of glucose in the second culture interval (days 3–5) is an absolute requirement for the efficient production of viable blastocysts (Gardner and Lane, 1997) and is consistent with the results from glucose uptake studies of human blastocysts in vitro (Hardy, 1993).

A potential role for glucose in fertilization has been suggested by several investigators. Quinn (1995) noted that more spermatozoa were required in glucose-free medium to achieve a fertilization rate equivalent to that of glucose-containing medium. Gardner and Lane (1997) likewise have recommended that either sperm number or glucose concentration be increased for fertilization in G1 medium to overcome the reduction in sperm velocity and fertilization reported by Quinn. Mahadevan et al. (1997) also indicated that fertilization and sperm movement is decreased in Ham’s F10 medium devoid of glucose compared to that medium containing glucose. No reduction in the fertilization rate was seen in P1 medium when compared to human tubal fluid (HTF) medium (Graham and Pool, 1998) or Ham’s F10 (unpublished observations). The decline in fertilization reported by others in glucose-free medium is therefore not attributable solely to the absence of glucose, but must involve other medium components, making the observed phenomenon medium-dependent. Alternatively, the taurine included in P1 medium may exert a positive influence on the fertilization process, thus masking any detrimental effects that might be mediated by the absence of glucose.

The exact role of inorganic phosphate (Pi) in preimplantation embryo development is not clear. The presence of Pi in mammalian embryo culture medium, however, has been associated with embryo arrest in vivo (Bavister, 1995). Matsumoto and Sugawara (1998) recently showed that short term exposure of Pi to rat embryos during the early cleavage stage (first to second cleavage) retarded embryo development and resulted in a reduced blastocyst development rate. Long-term exposure resulted in embryo arrest. Their experiments revealed that this inhibition of development was not due to inhibition of histone H1 kinase activity or maturation-promoting factor activity. Until further studies reveal the requirement or benefit of the presence of Pi in the early culture interval, we suggest its omission from the medium in the first culture phase (up to day 3).

Another problem solved by sequential culture systems is meeting the changing amino acid requirements of preimplantation embryos in culture. These requirements have been studied exhaustively in a mouse model system (Gardner and Lane, 1997). The most pregnancies following embryo transfer were obtained when medium for the first culture interval contained Eagle’s non-essential amino acids and the second medium contained all 20 Eagle’s amino acids, essential and non-essential (Lane and Gardner, 1997). Sequential culture of human embryos using this same approach has been successful (Gardner et al., 1997), although the exact amino acid requirement for human embryos has not been determined. In contrast to G1 medium, which contains Eagle’s non-essential amino acids, P1 medium contains only the single amino acid, taurine. Taurine has been shown, however, to promote embryogenesis to advanced stages in the hamster, even when it is included as the only amino acid in the medium (Bavister and McKiernon, 1993). We now show that medium containing only taurine as the sole amino acid can suffice as the first medium in a sequential system that includes a complex medium (blastocyst medium) as the second component.

As mentioned above, the exact requirements of amino acids and the roles they play in the early culture interval are not known for human embryos. However, there has been speculation that amino acids may function other than as nitrogen sources in the first interval, playing a more physical role, perhaps as osmolytes (Gardner and Lane, 1997). This is of particular interest since taurine has been shown to serve as an effective osmolyte in both human and mouse embryos. Dumoulin et al. (1997) preloaded oocytes and embryos with [3H]-taurine and showed that taurine is released by embryos that have to change cell volumes either by extracellularly-induced or intracellularly-occurring osmotic imbalances. As the authors indicate, its chemical inertness, lipophobic properties and ionic neutrality at physiological pH make taurine an ideal molecule to be retained within the cell at high concentrations, all of which underlie its suitability as an osmolyte. Taurine has also been described as having other protective functions, such as serving as an antioxidant. Li et al. (1993) have shown that the inclusion of taurine can increase the proportion of rabbit zygotes reaching the blastocyst stage and increase blastocyst cell number in protein-free medium to an extent equal to the inclusion of an optimal concentration of superoxide dismutase (600 IU/ml).

There are possibly other amino acids besides taurine that alone can promote embryogenesis to advanced stages. Devreker et al. (1998) measured an increase in the percentage of human embryos reaching the blastocyst stage when glutamine was included in a glucose-free variant of Earle’s Balanced Salt Solution, although the total cell number was not increased. The viability of these blastocysts upon transfer, unlike those produced with taurine in the first culture interval in the present study, has yet to be demonstrated.

What has become clear in studies both from animal models (Pollard et al., 1995; Lane and Gardner, 1997) and in humans (Gardner et al., 1998) is that a complex array of amino acids is required in the second culture interval to produce blastocysts efficiently. Pollard et al. (1995) compared the growth of pig zygotes in combinations of simple and complex media. Not only was a sequential approach of simple followed by complex medium superior to either medium used alone, but simple medium supplemented with whole serum in the second interval failed to produce as many hatching blastocysts as minimum essential medium. The blastocyst medium used in the present study not only contains a complex array of amino acids, but also vitamins and nucleic acid precursors. The necessity for these components has yet to be examined in our hands, although
their inclusion does not appear to compromise viability. Desai et al. (1997) also used a modified minimum essential medium (αMEM) to grow spare human embryos to blastocyst. Their medium was supplemented with 10% SSS as in this report. They reported a 37% blastocyst rate compared to the 54% we have using our sequential approach.

This report demonstrates that a simple system of sequential culture generated acceptable blastocyst development (54%) with ‘leftover’ embryos, without the use of feeder cells or conditioned medium. Recognizing the deferential metabolic requirements of early and late cleavage stage embryos has enabled the application of a glucose/phosphate-free simple culture medium (P1) for up to 72 h of culture and a complex glucose containing medium (blastocyst medium) for subsequent blastocyst development. In a small series of thawed and fresh blastocysts, we have demonstrated that the blastocysts derived from this culture system are viable and able to generate clinical pregnancies. A larger trial with fresh blastocyst transfer is currently underway.

References


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