Culture of viable human blastocysts in defined sequential serum-free media

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In human in-vitro fertilization (IVF), embryos are routinely transferred to the uterus on either day 2 or day 3 of development, resulting in a 10–15% implantation rate. However, in other mammalian species, the transfer of cleavage stage embryos, which normally reside in the oviduct, to the uterus results in a significantly lower implantation rate compared with blastocysts. It is therefore proposed that, in order to increase implantation rates in human IVF, one has to move to extended culture and transfer at the blastocyst stage. The transfer of blastocysts will not only help synchronize the embryo with the female tract but will facilitate the identification of those embryos with little or no developmental potential. In order to culture viable blastocysts it is important to use more than one culture medium to cater for the changing requirements of the preimplantation embryo as it develops and differentiates. If sequential culture media are not used, one can obtain blastocysts but their resultant viability is low. The use of sequential serum-free media in human IVF has resulted in >50% of embryos becoming blastocysts with an implantation rate of ~50%. Further advances in human embryo culture should come from the replacement of protein with the glycosaminoglycan hyaluronate, which is more suitable than albumin in supporting implantation in the mouse, and which will eliminate biological variation and possible contamination from blood products. With the routine culture of human blastocysts will come the introduction of non-invasive tests of embryo viability, capable of identifying those blastocysts most likely to develop from a given cohort. As the implantation rate of blastocysts is higher than that of the cleavage stage embryo, fewer embryos will be required for transfer in order to establish a successful pregnancy, thereby reducing the number of multiple gestations and increasing the overall efficiency of human IVF.

Key words: blastocyst transfer/human metabolism/viability

Introduction

There are considerable advantages of being able to culture the human embryo to the blastocyst stage before transfer in human in-vitro fertilization (IVF). Advantages
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include: (i) an increased implantation rate due to the identification of those embryos with little if any developmental potential and the synchronization of the embryo with the female tract; (ii) a decrease in the number of embryos required at the time of transfer in order to achieve a pregnancy; (iii) a decrease in the number of multiple gestations; (iv) an ability to undertake cleavage stage embryo biopsy without the need for cryopreservation when the biopsied blastomere has to be sent to a different locale for analysis; (v) facilitating the introduction of trophectoderm biopsy, which means the removal of non-embryonic tissue for analysis, which in itself has both ethical and religious implications; and (vi) allowing the introduction of quantitative methods of embryo viability assessment, as at the blastocyst stage embryo physiology is being analysed and not that of the oocyte (which is the case when the cleavage stage embryos are examined after their brief growth in vitro).

So why is the human embryo not routinely transferred at the blastocyst stage in human IVF? The main reason is that culture media have tended to be too simplistic, with conventional embryo culture media being based on a balanced salt solution with added carbohydrates as energy sources. At the other end of the spectrum several laboratories use tissue culture medium such as Ham’s F-10 or minimal essential medium (MEM), which were designed to support somatic cell lines in vitro, and do not reflect the actual requirements of the mammalian preimplantation embryo and may be considered too complex. The problem of which medium composition is the optimum has been further complicated by the use of a single medium in an attempt to support all stages of development. As the zygote and blastocyst have differing physiology and metabolism they subsequently have different requirements in culture. It is therefore proposed that in order to optimize embryo development in culture one has to formulate more than one culture medium to support embryo development in vitro.

The two key nutrients utilized by the developing embryo are carbohydrates and amino acids. The significance and role of these will be discussed in turn.

**Carbohydrates**

The human embryo undergoes a change in its nutrient preference as it develops, pyruvate being the preferred nutrient of the cleavage stage embryo, with glucose becoming increasingly utilized as development to the blastocyst proceeds (Hardy et al., 1989). Interestingly, such changes in carbohydrate uptake mirror the concentration of available nutrients within human oviduct and uterine fluids, in which pyruvate and lactate are at their highest concentration in the oviduct at the time when the embryo is present, while glucose concentration is reduced (0.5 mM). In contrast, the concentration of pyruvate and lactate are lower in the uterus, but glucose concentration is at its highest (3.15 mM) (Gardner et al., 1996a). In conventional embryo culture media, such as human tubal fluid (HTF) (Quinn et al., 1985), and Earle’s medium (Edwards, 1981), glucose can impair human embryo development in culture (Conaghan et al., 1993; Quinn, 1995).
An explanation for this observation has been derived from work on the embryos from other mammalian species. Glucose is not the preferred energy substrate of the cleavage stage embryo and yet it appears that in conventional embryo culture media, glucose is actually utilized by the cleavage stage embryo at the expense of oxidation (Menke and McLaren, 1970; Gardner, 1998a; Gardner and Leese, 1990; Seshagiri and Bavister, 1991; Gardner and Lane, 1993a; Gardner and Sakkas, 1993). This phenomenon is similar to that reported for certain types of tumour cell metabolism, and is known as the Crabtree effect (Koobs, 1972).

Importantly, however, this impairment of metabolic function is not evident when specific amino acids and the chelator EDTA are present in the culture medium. Both amino acids and EDTA act through independent mechanisms to suppress glycolytic activity in the early embryo (Gardner and Lane, 1993a; Lane and Gardner 1997a; Gardner 1998a). Furthermore, they act in synergy to minimize any adverse affect of glucose on the cleavage stage embryo. At the blastocyst stage the embryo can readily utilize glucose as both an oxidative and glycolytic energy source. The mechanisms underlying this metabolic switch have been documented elsewhere (Biggers et al., 1989, Leese, 1991; Rieger, 1992; Gardner 1998a,b).

However, two important considerations regarding the use of glucose by the embryo post compaction are worth discussing. Firstly glucose will have an important role in biosynthesis, as it is involved in the generation of ribose moieties for nucleic acid synthesis and the NADPH required for the biosynthesis of lipids and other complex macromolecules. Secondly, the work of Hewitson and Leese (1993) indicates that the inner cell mass generates its energy predominantly from glycolysis. Therefore a suppression of glycolysis by either substrate (glucose) limitation or the inhibition of an enzyme in the pathway (for example by EDTA), may well interfere with the development of the inner cell mass, thereby affecting subsequent fetal development. Indeed, this does appear to be the case. When mouse zygotes were cultured to the blastocyst stage in the absence of glucose, although the resultant blastocysts implanted in the uterus after transfer, significantly more fetuses were lost in comparison with the control blastocysts which had developed in the presence of glucose (Gardner and Lane, 1996). Similarly, if mouse embryos were cultured to the blastocyst stage in the continual presence of EDTA, which suppresses glycolysis, then resultant fetal development was significantly lower than from those blastocysts which had been exposed to EDTA for the first 48 h of culture from the zygote (Gardner and Lane, 1996). Gardner et al. (1997a) went on to demonstrate that the presence of EDTA in the culture medium for the first 72 h of culture significantly improved development of cattle embryos to the 8-cell stage. However, if the resultant 8-cell embryos were left in the presence of EDTA, then the developing blastocysts had a significantly smaller inner cell mass, and the number of cells in the trophectoderm was unaffected. Such data highlight the differing metabolism of the cleavage stage and post compaction embryo and supports the hypothesis that different culture conditions are required at different stages of development.
in order to satisfy the changing requirements of the embryo (Gardner and Lane, 1993b).

**Amino acids**

Until relatively recently, amino acids have not been considered to be important regulators of human embryo development. However, a resurgence of interest in their role in mammalian embryo development leads one now to conclude that amino acids are amongst the most important of regulators for the early embryo. The physiological basis for the inclusion of amino acids in embryo culture medium stems from the fact that amino acids are abundant within the fluid of the female reproductive tract (Miller and Schultz, 1987; Gardner and Leese, 1990; Moses *et al.*, 1997), and that the oocyte and embryo maintain an endogenous pool of amino acids through specific carriers on the plasma membrane (Schultz *et al.*, 1981; Miller and Schultz, 1987; Van Winkle, 1988). Interestingly, those amino acids which are most abundant in the oviduct are those with the greatest beneficial effect on the cleavage stage embryo (Bavister and McKiernan, 1993; Gardner and Lane, 1993c). These amino acids include; alanine, glutamate, glutamine, glycine, proline and serine. Together, these amino acids have been shown to significantly decrease the time that the mouse embryo takes to complete the first three cleavage divisions and undergo compaction (Lane and Gardner, 1997b), whilst decreasing the time it takes to form a blastocyst (Gardner and Lane, 1993c). This group of amino acids also shares a striking homology with those amino acids present in Eagle’s non-essential amino acids (Eagle, 1959), i.e. those amino acids not required by somatic cells in culture.

Importantly, those amino acids not present at high levels in the oviduct, which are classified as essential by Eagle (1959), confer no beneficial effect to the cleavage stage embryo (Gardner and Lane, 1993c; Lane and Gardner 1997c). Indeed, exposure of the mouse embryo to the essential amino acids, at the concentrations in Eagle’s medium, prior to the 8-cell stage results in a loss of viability (Lane and Gardner, 1994). Interestingly, however, like the switch the embryo undergoes regarding its utilization of carbohydrates, there is also a switch in the requirements for amino acids. This switch seems to stem specifically from the different requirements of the two cell types in the blastocyst. The inner cell mass appears to require the essential group of amino acids (i.e. those required by somatic cells in culture), whilst the trophectoderm utilizes the non-essential amino acids and glutamine (Lane and Gardner, 1997c). The reason for this observed switch in amino acids has been attributed to the roles that specific amino acids have in embryo physiology (Gardner and Lane, 1997; Gardner 1998a; 1998b). In essence the non-essential amino acids serve as regulators of energy metabolism, osmolytes and intracellular pH buffers prior to compaction. Such data like that obtained for the utilization of carbohydrates support the hypothesis that more than one culture medium is required for the optimal development of the preimplantation embryo in culture.
Development of sequential culture media

In light of the dynamics of embryo physiology and the resultant changes in requirements for both carbohydrates and amino acids, it is proposed that optimal development of the mammalian embryo in culture will require more than a single culture medium. Rather, two or more will be required to meet the changing requirements of the embryo. To this end, two culture media, designated growth 1 and growth 2 (Gl and G2) were formulated to support the growth of the human pronuclear embryo to the blastocyst stage (Gardner, 1994; Barnes et al., 1995; Gardner and Lane, 1997). The Gl medium is based on the levels of carbohydrates present in the human Fallopian tube at the time when the cleavage stage embryo is present. This medium also contains those amino acids which have been shown to stimulate development of the cleavage stage embryo (i.e. the non-essential amino acids and glutamine). The chelator EDTA is also present, not only to sequester any toxic divalent cations present in the system, but also to help minimize glycolytic activity of the embryo, thereby minimizing metabolic perturbations.

In contrast, G2 medium is based on the levels of carbohydrates present in the human uterus and contains both non-essential and essential amino acids to facilitate both blastocyst development and differentiation. EDTA is not present in medium G2 as it appears to selectively impair inner cell mass development and function, culminating in a loss of viability (Gardner and Lane, 1996; Gardner et al., 1997a). Both Gl and G2 media are supplemented with serum albumin. Serum is not required nor desired in embryo culture systems, especially those designed to support blastocyst growth (Gardner and Lane, 1993b; Gardner, 1994). The beneficial effects of using sequential media to support mouse embryo development in culture are shown in Figure 1. One of the most important things to come from this work is the observation that it is possible to generate healthy looking blastocysts in culture which unfortunately have little if any further developmental potential. This stems from the fact that different components of the culture system affect different aspects of embryo development. When mouse embryos are cultured in Gl medium for the entire preimplantation period to the blastocyst, although the embryos form healthy looking blastocysts, most implantations are lost, i.e. they did not have a sufficient inner cell mass to form a viable fetus (Gardner et al., 1997b; Lane and Gardner, 1997c). The lack of adequate inner cell mass development stems from both the lack of sufficient glucose and the presence of EDTA (both affecting glycolysis) and the omission of essential amino acids. In contrast, those embryos that were switched to G2 medium after 48 h of culture formed blastocysts at the same rate and of equivalent morphologies, but due to the development of a significant inner cell mass, very few implantations were lost thereby maintaining a very high pregnancy rate.

The observation that a well-formed blastocyst does not necessarily equate to a viable blastocyst is an important one, and one which can perhaps explain why previous attempts to culture the human embryo to the blastocyst stage have resulted in low implantation rates after transfer. For example, Bolton et al. (1991)
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Figure 1. Effect of sequential culture media on the development of F₁ (C57BL/6×CBA/Ca) mouse zygotes in vitro. Zygotes were collected at 20 h post-administration of human chorionic gonadotrophin (HCG). All media were supplemented with bovine serum albumin (BSA, 2 mg/ml). All embryos were transferred to fresh medium after 48 h of culture, with the exception of embryos in medium G1, where the embryos were transferred to either medium G1 or G2. To compensate for this, twice the number of embryos were originally cultured in medium G1, although only a designated 50% of these embryos were used in the statistical analysis of the 44–52 h data set. (a) Embryo cell number after 44, 48 and 52 h of culture. Values are means ± SEM. n = 200 embryos/medium. Media: G1 (solid bar); human tubal fluid (HTF; open bar); Ham’s F-10 (hatched bar). **Significantly different from other media, P <0.01. (b) Embryo development after 72 h of culture. n = 150 embryos/medium. G1/G2; embryos cultured for 48 h in medium G1 and then transferred to medium G2. Blastocyst (solid bar), hatching blastocysts (as a percentage of total blastocysts; open bar). Like pairs are significantly different; ab, cd P <0.05; bd P <0.01. (c) Embryo development after 92 h of culture, n = 150 embryos/medium. G1/G2; embryos cultured for 48 h in medium G1 and then transferred to medium G2. Blastocyst (solid bar), hatching blastocysts (as a percentage of total blastocysts; open bar). Like pairs are significantly different; a, c, d P <0.05. Significantly different from medium G1 and G1/G2; **P <0.01. (d) Cell allocation in the blastocyst after 92 h of culture. n = 150 embryos/medium. G1/G2; embryos cultured for 48 h in medium G1 and then transferred to medium G2. Trophoderm (solid bars), inner cell mass (open bars). Significantly different from other media; *P <0.05; **P <0.01. (e) Viability of cultured blastocysts; n = at least 60 blastocysts transferred per treatment. G1/G2; embryos cultured for 48 h in medium G1 and then transferred to medium G2. Implantation (solid bar), fetal development per implantation (open bar). Like pairs are significantly different; a, d P <0.05; b, c P <0.01

obtained a very respectable 40% rate of blastocyst development from human pronuclear embryos. However, the resultant implantation and pregnancy rate was only 7%. The medium used in this study was Earle’s supplemented with pyruvate and 10% maternal serum. Clearly then, such media are not suitable for extended human embryo culture.
This approach of using sequential culture media has now been applied in pilot clinical studies at two IVF programmes, one in Colorado, USA (Colorado Center for Reproductive Medicine) and one in Melbourne, Australia (Monash IVF). In both programmes the use of sequential embryo culture media has resulted in blastocyst development of $\geq 50\%$ with a significant increase in subsequent implantation rate (Gardner et al., 1997b, 1998; Jones et al., 1998). Data to date at the Colorado Center for Reproductive Medicine after 50 blastocyst transfers show that an implantation rate of $\sim 50\%$ can be achieved, resulting in an ongoing pregnancy rate of 70% with a mean of 2.6 blastocyst transferred. Importantly, all the babies born from this culture system are normal. Clearly, now that culture systems have been tested and the viability of the resultant blastocysts established, it is important to reduce the number of embryos transferred down to two, the ultimate goal being the transfer of a single blastocyst. Large-scale randomized trials are now required to validate the findings of these pilot studies.

There remain two important issues regarding human embryo culture and transfer in human IVF; the use of blood products in assisted conception and the need to identify the most viable embryos for transfer.

**Alternatives to protein in embryo culture medium**

There is a possible risk of infection arising from the use of blood products in IVF. Not only does the use of pooled blood to generate serum albumin always represent a cause for concern, but batches of serum albumin vary significantly in their embryotrophic ability, thus making batch to batch comparisons very difficult and highlighting the problem of biological variation. This very problem has led to the search for alternative macromolecules to serum albumin. The synthetic polymers polyvinylalcohol and polyvinylpyrrolidone have both been used in assisted reproductive technologies (Bavister, 1995). However, neither can be considered as a physiological alternative to protein, and the teratological nature of these compounds has not been fully evaluated. Furthermore, the true role of serum albumin in embryo culture has yet to be fully elucidated, although it is plausible that albumin helps to bind and stabilize growth factors.

The presence of macromolecules in an embryo culture system serves to facilitate manipulation of gametes and embryos, which would otherwise adhere to the plastic culture vessel. A possible alternative to albumin is the glycosaminoglycan, hyaluronate. Not only do the levels of hyaluronate increase in the uterus around the time of implantation (Zorn et al., 1995), but the human embryo expresses the receptor for hyaluronate throughout preimplantation development (Campbell et al., 1995). Although hyaluronate is a glycosaminoglycan, unlike other glycosaminoglycans such as heparin, it has no protein moieties and can therefore be considered as a polysaccharide. This, therefore, removes both the problem of variation and contamination, as hyaluronate can be synthesized in a pure form. In preliminary trials of hyaluronate in mouse embryo culture and transfer, it was found that not only could hyaluronate (0.5 mg/ml) readily replace...
serum albumin for in-vitro cultures, but more importantly it significantly increased the implantation rate of resultant blastocysts (Gardner et al., 1997c). Hyaluronate supported a significantly higher implantation rate than albumin. Furthermore, this increase in implantation rate could be attributed to the transfer medium alone.

When embryos were cultured in appropriate sequential media similar to G1 and G2, there was no evident benefit in vitro from the presence of any macromolecule/protein. Embryos formed blastocysts at the same rate in medium devoid of any macromolecule, and the blastocysts had inner cell mass and trophectoderm cells equivalent to those embryos cultured with either albumin or hyaluronate. However, when embryos were pre-equilibrated in medium supplemented with 0.5 mg/ml hyaluronate for 5 min prior to transfer in medium containing hyaluronate, the resultant implantation rate was equivalent to that obtained for embryos cultured for the entire preimplantation period in the presence of hyaluronate (Gardner et al., 1997c).

The beneficial effect of hyaluronate on the embryo and implantation may be attributed to one or more roles of hyaluronate; these include the ability to form an anti-viral and anti-immunogenic layer around the embryo, an ability to increase angiogenesis and perhaps most importantly to facilitate the rapid diffusion of the contents of the transfer medium (the embryo) into the fluid of the uterus. As uterine fluid is a viscous solution, the transfer of a relatively aqueous solution, such as culture medium with albumin, to the uterine lumen will result in the slow mixing of the medium and embryo with the luminal contents. In contrast, the transfer of an embryo in a hyaluronate solution will facilitate diffusion of the embryo into the luminal environment. Furthermore, hyaluronate may be involved in the initial phases of attachment of the blastocyst to the endometrium.

**Development of blastocyst viability assays**

Conventional embryo culture media induce considerable cellular trauma in the developing embryo. One of the key manifestations of this is an abnormal pattern of energy metabolism. As the developing embryo requires sufficient levels of cellular energy for DNA replication, biosynthesis and mitosis, any aberrations in the embryo’s metabolism has dire consequences for subsequent development and viability. Studies on both cattle and mice have determined that there is a relationship between both the rate and normality of nutrient utilization and developmental potential. Renard et al. (1980) observed that day 10 cattle blastocysts with a glucose uptake >5 μg/h developed better in culture and gave rise to more pregnancies than blastocysts with a glucose uptake <5 μg/h. Subsequently, Gardner and Leese (1987) used the non-invasive technique of ultramicrofluorescence to measure glucose uptake by individual day 4 mouse blastocysts prior to their transfer to recipient females. Those blastocysts that went to term had a significantly higher glucose uptake in culture than those embryos that failed to develop after transfer. Unfortunately these studies were
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retrospective and as such could not conclusively demonstrate whether it was possible to identify viable embryos prior to transfer using metabolism as a marker. However, a study of the metabolism of day 7 cattle blastocysts before and after cryopreservation showed that it was possible to identify those blastocysts capable of re-expansion in the hours immediately post-thaw. Those blastocysts which survived the freeze–thaw procedure had a significantly higher glucose uptake and lactate production than those embryos which did not re-expand and subsequently died (Gardner et al., 1996b). Of greater significance however, was the observation that there was no overlap in the distribution of glucose uptake by the viable and non-viable embryos, suggesting that it may therefore be possible to use metabolic criteria for prospective selection of viable embryos (Gardner et al., 1996b). Following on from this study, Lane and Gardner (1996) performed a prospective trial in which day 5 mouse blastocysts were classified as either viable or non-viable according to their glycolytic activity. It was found that those blastocysts which exhibited a pattern of glycolytic utilization similar to that of embryos developed in vivo had a developmental potential of 80%, whilst those blastocysts which exhibited an excessive lactate production (i.e. aberrant glycolytic activity), had a developmental potential of only 6%. As such, this data supports the hypothesis proposed by Rieger (1984) that embryonic metabolism can be used to successfully identify viable embryos in culture prior to transfer, thereby significantly increasing pregnancy rates. Now that the routine culture of the human embryo to the blastocyst stage is achievable, non-invasive assessment of blastocyst metabolism may be used to identify those embryos with the highest developmental potential prior to transfer.

Conclusions

Using sequential serum-free culture media it is possible to culture the human pronuclear embryo to the blastocyst at acceptable frequencies. More importantly, using this approach the resultant blastocysts have a high implantation rate, culminating in a high pregnancy rate and significantly reducing the need for the transfer of multiple embryos, thereby reducing the number of multiple gestations.

The next generation of embryo culture media are likely to be protein free, using such macromolecules as hyaluronate as a physiological alternative to serum albumin. Such media will have inherently less biological variation and should significantly reduce the potential for infection in the culture system.

With the advent of blastocyst culture and transfer in human IVF, it should be feasible to further reduce the number of embryos transferred whilst maintaining high pregnancy rates, by the implementation of non-invasive methods of assessing embryo metabolism prior to transfer.

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