Improved methods for blastocyst formation and culture

Yves Ménézo1-4, Anna Veiga2 and Moncef Benkhalifa1

1Laboratoire de Cytogenétique et de Procréation Médicalement Assistée, Laboratoire Marcel Mérieux, 1 Rue Laborde, Bron, France
2IVF Laboratory, Departement of Obstetrics and Gynecology, Institut Dexeus, Barcelona, Spain, and 3Unité de Biologie du Développement Pré-implantatoire, INSA, Biologie 406, 69621 Villeurbanne cedex, France
4To whom correspondence should be addressed at Laboratoire de Cytogenétique et de Procréation Médicalement Assistée, Laboratoire Marcel Mérieux, 1 Rue Laborde, Bron, France

Transfer at the blastocyst stage has been proposed to increase the pregnancy rates after in-vitro fertilization (IVF) and embryo transfer. In the first period of experiments, culture in a single medium, from fertilization to blastocyst led to disappointing results: low blastocyst formation rates and low implantation rates per blastocyst transferred. Then the period of co-culture began (starting with animals in the early 1980s and with humans in the early 1990s). With this technique, using tubal or granulosa cells or layers obtained from established cell lines of transport epithelium origin, blastocyst formation has reached ~50% and the overall implantation rate is ~25%. The embryos obtained have high numbers of cells (>200 cells for a day 6 expanded blastocyst). Co-culture with fibroblasts has been found to be useless. This technology has been proven reliable and reproducible: Blastocyst formation is highly dependent on maternal and paternal factors. It has enabled the design of efficient freezing and thawing protocols. Numerous interesting observations have been obtained to reach the third period, i.e. the use of sequential media. A simple medium is used for fertilization, then another one is used from fertilization up to the 4-cell stage (beginning of waves of transcription), then a third medium is used for development up to the blastocyst stage. The results obtained seem very similar to the one obtained with co-culture. Obviously it is now time, in humans, to switch to sequential media.

Key words: culture medium/embryo/IVF

Introduction

The goal of in-vitro fertilization (IVF) and embryo culture is to provide high quality embryos capable of continued development and implantation, and resulting in viable births. Since the initial embryo culture studies, considerable progress
has been made in culturing preimplantation embryos. With others (Tervit et al., 1972), we started in the early seventies to design and define new, more complex culture media, based on the composition of genital tract secretions (Ménézo, 1976). Despite our efforts, retarded development occurs in vitro (human, Bolton et al., 1989). This leads to developmental arrest at the time of genomic activation (Poueymirou et al., 1989). Before genomic activation, there is no transcriptional activity. So at the end of maturation, the oocyte has to contain all proteins and/or transcripts coding for all the enzymes required for all the metabolic pathways and at the right equilibrium. Moreover, the cycle of zygotic gene activation is the longest of preimplantation development: any delay at this time will result in a decrease in the level of mRNA below critical thresholds. Moreover, culture conditions have a direct impact on transcription and translation (Jung, 1989; Ho et al., 1994), the metabolism of the embryo is depressed in vitro (Leese, 1995) and protein turnover is accelerated (Jung, 1989). A decrease in viability (human, Bolton et al., 1989; mouse, Bowman and McLaren, 1970), and cell number (human, Vlad et al., 1996) has also been observed. The impact of lower cell numbers is evident at the time of hatching: trophectoderm cells are responsible, at least in part, for zona pellucida lysis.

All these problems are the result of suboptimal culture conditions. An on-time blastocyst formation is a first indicator of embryo quality, the ultimate control being live birth rates after transfer. There are obviously dynamic changes in the embryo environment during its transit through the oviduct and then through the uterus (Rousseau and Ménézo, 1993). Two solutions have been proposed to fit with these requirements of the embryo: (i) sequential changes in the composition of the culture media; and (ii) dynamic changes by using co-cultures. We have tried to take into account studies where the normality of culture conditions have been validated by embryo transfers, but this is very rare.

Co-culture

Organ culture, the first co-culture system based on a paracrine effect, was developed in the mouse by Biggers et al. (1962), who cultured embryos in hormonally primed oviducts. Other experiments, based on an autocrine effect, were successfully performed with bovine and ovine embryos co-cultured with bovine trophoblastic tissue (Camous et al., 1984). Live calves were obtained after >5 days of co-culture. Subsequently, we demonstrated that a genital origin for the cells was not necessary for culture of human preimplantation embryos (Ménézo et al., 1990).

The co-culture technique is totally different from classical embryo culture technologies. The choice of medium for embryo co-culture studies is critical: the co-culture medium has to fit with embryo and feeder cell needs. False positive and false negative effects may be immediately linked to the co-culture medium (Xu et al., 1992), and the use of different culture media, with the same cell feeders, can lead to dramatic variations in results. In the same way, the presentation
of the cells (monolayers, cell suspension) can interfere with the outcome of co-culture.

Co-cultured embryos usually have higher cell numbers than do those cultured in simple culture media (Goodeaux et al., 1989; Vlad et al., 1996). Two recent randomized experiments (Van Blerkom, 1993; Vlad et al., 1996) demonstrated the importance of the technical aspects for co-culture of human embryos. The mean cell numbers in the control groups were the same, but they were totally different in the experimental groups (Table I), showing that for the same experimental protocol opposite observations were described. The control groups, with no cells, were performed with a single culture medium which had not really been designed for this purpose. The idea of a single culture medium for obtaining blastocysts without feeder cells is obsolete. Directly related to the cell number, organ culture allows the embryo to recover a dry matter weight similar to the in-vivo situation after a critical point, corresponding to the zygotic gene activation (Turner et al., 1994).

An important aspect of cell feeder action is the interaction with embryo metabolic pathways. There are deficits in enzyme and messenger mRNA, or inhibition of pathways (see phosphofructokinase: Barbehenn et al., 1974) or de-equilibrated enzymes (e.g. glucose phosphate isomerase, Ménézo and Khatchadourian, 1990). This results in the impossibility of metabolizing some compounds, the result being a waste of energy and an accumulation of useless compounds. This is demonstrated in the case of glucose: high external concentrations, in conventional culture media leads in vitro to the accumulation of glycogen in the mouse. This does not occur in vivo and/or in co-culture (Khurana and Wales, 1987). It is more than probable that the metabolism of feeder cells supplies small molecules allowing the cell machinery to work with maximum efficiency. The lactate/pyruvate ratio is probably optimized as well (Ouhibi et al., 1990). A marginal drop of glucose in the embryo environment is due to the feeder metabolism (Ouhibi et al., 1990; Edwards et al., 1997), but in no cases do the glucose concentrations drop below mM levels. Glucose is necessary even

Table I. Cell numbers in human blastocysts

<table>
<thead>
<tr>
<th></th>
<th>Simple medium</th>
<th>Co-culture</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>D5.5</td>
<td>46.5</td>
<td>45</td>
<td>Van Blerkom, 1993</td>
</tr>
<tr>
<td>EBSS + 15% serum</td>
<td>± Vero</td>
<td></td>
<td>Vlad et al., 1996</td>
</tr>
<tr>
<td>D6</td>
<td>43</td>
<td>120</td>
<td>Hardy et al., 1989</td>
</tr>
<tr>
<td>EBSS + 15% serum</td>
<td>± Vero</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>D6 Expanded</td>
<td>82</td>
<td>ND</td>
<td>Ménézo et al., 1993</td>
</tr>
<tr>
<td>T6 or Earle</td>
<td></td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>D6 expanded</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2 + Vero</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND = not determined.
EBSS = Earle’s balanced salt solution.
Improved methods for blastocyst formation and culture

at early stages. Obviously, glucose and glutamine are present in vivo in the embryo environment during its transit (Rousseau and Ménézo, 1993).

There is a contribution of amino acids by the feeder leading to a better equilibrium of amino acids, allowing for their efficient uptake. Co-culture provides a good redox potential (equilibrium between reducing and oxidative substances in favour of reducing activity), which is rarely taken into account. Feeder cells synthesize and release reducing substances such as glutathione and in particular hypotaurine, which are efficient for improving early preimplantation embryo development (Barnett and Bavister, 1992). A specific enzymatic system is responsible for its synthesis in the embryo environment, namely the cysteine sulphinate decarboxylase system (Guérin and Ménézo, 1995). These reducing compounds are necessary to avoid undesirable oxidation of lipids and other compounds such as cysteine and methionine.

However, amino acids can be toxic through their catabolism and the formation of ammonium ions (Gardner and Lane, 1993). In vitro, in open conditions, ammonia forms ammonium carbonate and/or bicarbonate, which are highly unstable, especially in alkaline pH. Ammonia is eliminated or recycled enzymatically in vivo (Ménézo et al., 1993; Lane and Gardner, 1995), or in co-culture, through formation of alanine, glutamine and asparagine.

Macromolecules

No specific embryotrophic factors secreted by feeder layers have yet been identified. Blastocyst formation can be obtained with serum albumin and serum fractions, inappropriately called synthetic serum substitute (Desai et al., 1997); obviously serum is not necessary in IVF (Ménézo et al., 1984; Bavister, 1995; Laverge et al., 1997).

It is more than probable that the ‘trophic’ factors are ubiquitous. Additionally, embryo development is significantly better when embryos are cultured in groups rather than alone. These results suggest an autocrine effect of trophectoderm on embryos (Camous et al., 1984) and between embryos (mouse: Paria and Dey, 1990). Growth factor receptors such as insulin-like growth factors I and II (IGF-I and IGF-II), insulin, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (Adamson, 1993) are present in early embryos. Transforming growth factor β and basic fibroblast growth factor synergistically promote early bovine embryo development during the fourth cell cycle (Larson et al., 1992). Growth factors are potential candidates for embryo autocrine effects, however it is not obvious that growth factors can work through an internal loop, even if the genes for the ligands and the growth factors are expressed (Watson et al., 1992). Chia et al. (1993) demonstrated temporal and spatial shut off of growth factors (EGF) and their receptors in the human blastocyst. This implies a need for an exogenous source of growth factors acting in a paracrine way. We demonstrated the release of EGF by Vero and bovine kidney (MDBK) cells as well as by oviduct cells. However there are, for oviduct cells, quantitative variations according to the species and to the number of subpassages. Growth
factors act synergistically (bovine: Thibodeaux et al., 1993) and if one pathway is blocked, another one is activated to by-pass the block.

Endocytosis, by the embryo, of cell cycle proteins from the feeder seems very improbable. Co-culture cells that express leukaemia inhibitory factor (LIF), such as Vero cells or uterine epithelial cells, enhance mouse blastocyst development in vitro (Kauma and Matt, 1995). LIF improves in-vitro development of ovine (Fry et al., 1992) and bovine embryos (Fukui et al., 1994). The role of LIF in human embryo development and implantation is not yet evident.

**Sequential media**

The progress brought by co-culture, and in general embryo biochemistry, and the concern about co-culture even with highly controlled cell lines, has pushed the use of cell free culture conditions (Gardner, 1994). There was a need for changes in concepts and formula of classical culture media. To follow the embryo’s needs, it is now obvious that a first medium has to be used before zygotic gene activation and one after.

Amino acids are necessary very early after fertilization (Gardner and Lane, 1996). They are even necessary for short-term embryo handling. The use of 5% O₂ may increase blastocyst formation. It is the high external concentration of glucose in too simple a culture medium which is toxic rather than the glucose *per se*. Neither Ali et al. (1993), nor Gardner and Lane (1996), were able to demonstrate any inhibitory effect of glucose when glutamine and/or other amino acids are present in the culture medium. The present trend of removing glucose and phosphate from mammalian embryo culture media seems to be non-physiological, replacing one artefact with another. However, in several IVF media, the level of glucose is severely reduced and phosphate is totally removed (Quinn, 1995). On the contrary, pyruvate is an interesting compound, acting not only as an energy source. Pyruvate is able to detoxify the ammonia in the embryo, through transamination and export of the alanine formed (Figure 1).

Glycine, which is the most abundant amino acid in the female genital tract (up to 5 mM), is catabolized into glycolate and glyoxylate (Khatchadourian et al., 1994), with a release of ammonia. Ammonia accumulation is typically an artefact of culture in microdrops under oil. That is why care should be taken concerning this point. Glycine acts synergistically with taurine to improve culture conditions. Glutamine is also needed before genomic activation.

At this point, serum is not necessary, but albumin is. Albumin is such a complex carrier of many kinds of molecules, that it is difficult to define a precise role; but its capacity to bind lipids is probably important.

EDTA is added in the first step of culture as a free radical scavenger through the elimination of Fe and Cu. EDTA is rather deleterious after genomic activation and should be removed in the second phase of culture. Growth factors are usually added for the second part of culture. Insulin, which has a positive effect on protein metabolism, and EGF are important. However, it is difficult to know if
mixture of growth factors are added since the composition of the culture media is not ‘open’.

Figure 1. Mechanism of ammonia removal by the embryo.

Comparative analysis of the results

Considering the latest results with sequential media (Bertheusen et al., 1997; Chouteau et al., 1998; Jones et al., 1998), it is clear that they should be taken into account. Paternal and the maternal (age) effects may interfere severely with all the yields. This type of analysis has not yet been made for sequential media, but a difference in the results would be surprising. Blastocyst formation yield is ~50%. From the remaining 50%, one half of the embryos is blocked, whatever the culture conditions, due to cytogenetic reasons (Benkhalifa et al., 1996). Table II gives the comparative results with the two techniques. An implantation rate per blastocyst of ~25% can be expected whatever the technique.

One point has to be further considered: the freezing ability of the blastocysts obtained. In our laboratory, the take home baby rate per frozen blastocyst, after co-culture is 10% (Table III). Obviously, this point has to be evaluated for blastocysts resulting from sequential culture conditions.

Conclusions

The low success rates for the culture of human and animal embryos in simple or complex media severely compromises the application of new technologies.
Y. Ménézo, A. Veiga and M. Benkhalifa

Table II. Blastocyst formations, transfer rates and implantation rates per blastocyst after co-culture or the use of sequential media

<table>
<thead>
<tr>
<th></th>
<th>Transfer rate (%)</th>
<th>Blastocyst formation (%)</th>
<th>Implantation rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-culture</td>
<td>92(^a)</td>
<td>45(^a)</td>
<td>20–25(^a)</td>
</tr>
<tr>
<td>Sequential media</td>
<td>78(^c)–96(^b,d,e)</td>
<td>45(^b,c,d,e)</td>
<td>23(^b,d,e)–36(^c)</td>
</tr>
</tbody>
</table>

\(^a\)Ménézo et al., 1993.  
\(^b\)Gardner and Lane, 1997.  
\(^c\)Bertheussen et al., 1997.  
\(^d\)Chouteau et al., 1997.  
\(^e\)Jones et al., 1998.

Table III. The two thawing protocols for transfers of co-cultured frozen blastocysts\(^a\). Figures in parentheses are percentages

<table>
<thead>
<tr>
<th></th>
<th>Stepwise</th>
<th>Two-step (no glycerol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thawing cycles</td>
<td>563</td>
<td>253</td>
</tr>
<tr>
<td>Transfer cycles</td>
<td>516 (92)</td>
<td>222 (87)</td>
</tr>
<tr>
<td>No thawed blastocysts</td>
<td>1239</td>
<td>534</td>
</tr>
<tr>
<td>No transferred blastocysts</td>
<td>1033 (83)</td>
<td>380 (71.2)(^b)</td>
</tr>
<tr>
<td>Clinical pregnancy/transfer</td>
<td>112 (21.7)</td>
<td>64 (29)</td>
</tr>
<tr>
<td>Ongoing pregnancy/transfer</td>
<td>98 (19)</td>
<td>49 (22.1)</td>
</tr>
<tr>
<td>Clinical implantations/embryo</td>
<td>138 (13.4)</td>
<td>67 (17.6)</td>
</tr>
<tr>
<td>Ongoing implantations/embryo</td>
<td>124 (12)</td>
<td>52 (13.7)</td>
</tr>
<tr>
<td>Live birth/frozen blastocyst (%)</td>
<td>10</td>
<td>9.81</td>
</tr>
</tbody>
</table>

\(^a\)Cryoprotectant addition is carried out in two steps: 5 and 9% glycerol (v/v).  
\(^b\)Significant (\(P < 0.001\)).

Co-culture of both mammalian and human embryos with somatic cells has been reported to promote the improved quality of embryos, the proportion of embryos cleaving to the blastocyst and hatched blastocyst stages, higher cell number and higher implantation and pregnancy rates after transfer (Bongso et al., 1991; Freeman et al., 1995). Cytogenetic selection in vitro is beneficial (Benkhalifa et al., 1996), and co-culture improves freezing efficiency.

The empirical approach of comparing the different formulae of culture media was ineffective until we understood the majority of the interactions in embryo metabolism. It is important to control, before and during embryo culture, the interactions of the different compounds with the gas phase and between each other. Now, sequential media are effective in human, with yields similar to that observed for co-culture, but the number of patients is still rather low. The time for extended controlled trials has now arrived.

In all cases, in order to evaluate new techniques and/or new culture media, embryos grown in vitro have to be transferred, to avoid any misleading observations, based only on morphology. Obviously, considering the early developmental arrests, transfers at early stages are too blind processes. The question is no longer co-culture or not, but the gaining of viable blastocysts. Transfers at the blastocyst stage, in IVF practice, should not be the exception.
but the rule: the ultimate goal being the improvement of assisted reproductive techniques, lessening the likelihood of multiple pregnancies.

References

Y. Ménézo, A. Veiga and M. Benkhalifa


Improved methods for blastocyst formation and culture


