Use of co-culture of human embryos on Vero cells to improve clinical implantation rate

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Co-culture of human embryos (n = 384 cycles) to the blastocyst stage using Vero cell monolayers was carried out between August 1995 and December 1997. A total of 2868 zygotes were co-cultured and 1027 embryos reached the blastocyst stage (blastocyst formation rate 35.8%). The blastocysts were frozen in 43.7% of patients. A mean of 1.8 blastocysts was transferred per patient and 95 pregnancies were obtained (pregnancy rate/cycle 24.7%). The blastocyst implantation rate was 23.6%. Miscarriage occurred in 15 patients (15.7%) and ectopic pregnancy in three (3.1%) patients. The multiple pregnancy rate was 32.6%. No differences were observed in the blastocyst rate between poor, normal or high response patients. Blastocyst formation was significantly lower when frozen donor spermatozoa were used. Significantly higher pregnancy rates per transfer and blastocyst implantation rates were attained when embryos were transferred on days 5 or 6 compared with day 7. No advantage was observed when co-culture was used in first cycle IVF patients, in comparison with conventional day 2 replacements. The use of blastocysts for preimplantation genetic diagnosis (PGD) increases the diagnostic reliability and widens diagnostic possibilities. A total of 215 cycles with frozen–thawed co-cultured blastocysts were carried out, with a pregnancy rate of 22.7% per replacement.

Key words: blastocyst transfer/co-culture/frozen–thawed blastocyst transfer/implantation rates/pre-implantation genetic diagnosis

Introduction

In-vitro preimplantation embryo development is characterized by retarded growth rates and loss of viability. Most in-vitro developmental arrests occur at the time of genomic activation, at the 6–8-cell stage, and are directly correlated with chromosomal anomalies (Ménézo and Ben Khalifa, 1995; Munné et al., 1995). Although the exact requirements for correct embryonic development have not yet been clearly established, in a high percentage of patients, two, three or four embryos of apparently normal morphology are replaced into the uterus. When compared with normal conditions in vivo, embryo transfer after in-vitro fertilization (IVF) is usually performed 24–48 h before the embryo is supposed to reach the uterine cavity. Attempts to improve embryonic–endometrial synchronization by extending the period of in-vitro culture have not been very successful. Most simple culture media used in IVF laboratories allow blastocyst development in only 20–25% of embryos under optimal embryo culture conditions (Bolton et al., 1991), although promising improvements have been reported recently using sequential media (Bertheussen et al., 1997; Gardner et al., 1998; Gardner, 1999; Jones et al., 1998; Ménézo et al., 1998a,b).

Co-culture has been widely used in animals with successful results (Iritani, 1988). Co-culture with trophoblastic cells allowed live births in the cow for the first time (Camous et al., 1984; Heyman et al., 1987). Oviduct cells have been used for embryo co-culture in sheep and cattle (Gandolfi and Moor, 1987; Eyestone and First, 1989).

Co-culture techniques have been used in human IVF and have shown encouraging results, sug-
gesting that the feeder cells improve embryo morphology, while more cells per embryo and a better defined inner cell mass (ICM) are obtained (Ménézo et al., 1992a; Vlad et al., 1996).

A range of different feeder layers including monolayers of established cell lines, such as Madin–Darby bovine kidney cells (MDBK) and African green monkey kidney epithelial cells (Vero) (Ménézo et al., 1986), oviductal epithelial cells of several species (Bongso et al., 1989; Ouhibi et al., 1989, 1990), fetal bovine uterine fibroblasts (Wiemer et al., 1989), human granulosa cells (Plachot et al., 1993; Quinn, 1994), and endometrial cells (Jayot et al., 1995) have been used in different studies. The mechanisms by which co-culture exerts its effects are not well understood. Two possibilities have been suggested: (i) removal of toxic components from the culture medium (heavy metal divalent cations and metabolic inhibitors); and/or (ii) by contribution of embryotrophic regulating compounds (small metabolites and growth factors) (Bongso et al., 1990, 1991, 1993).

In a previous study (Desai and Goldfarb, 1998), the concentration of interleukin-6 and platelet-derived growth factor (PDGF) increased in Vero cell culture supernatants during the culture period. When analysing medium supernatants that had contained embryos (at the time of transfer) a wide patient-to-patient variation was observed, indicating that co-cultured embryos are exposed to a dynamic environment.

Established cell lines (such as MDBK or Vero cells) checked for viruses, bacteria and other possible contaminants can be obtained commercially, either frozen or as monolayers, thus providing safe culture conditions.

Our results with the use of co-culture of human embryos on Vero cells in the IVF programme of Institut Universitari Dexeus from August 1995 to December 1997 are presented here.

Material and methods
Since August 1995, co-culture on monolayers of Vero cells has been included in the IVF programme of Institut Dexeus. We report our experience with this system from August 1995 to December 1997. The study group consists of 384 cycles with a mean patient age of 35.8 years (range 19–48 years). Patients were selected for inclusion on the basis of repeated implantation failure in previous IVF cycles. They had already undergone a mean of 3.0 embryo transfers (range 1–14) with a mean of 8.2 embryos per transfer (range 1–36). Infertility was due to tubal factor (32%), male factor (33%), endometriosis (10%), failed artificial insemination by donor (AID) (5%) and unexplained infertility (11%). Approval for the clinical application of coculture was obtained from the Ethical Committee of Institut Universitari Dexeus.

Co-cultures were prepared as described previously (Ménézo et al., 1990; Veiga et al., 1995). Briefly, frozen Vero cells (ATCC, MD, USA) in a cryotube (~10^6 cells/ml) were seeded in tissue culture flasks containing BM1 medium (Ellios Bio Media, Paris, France) supplemented with newborn calf serum (Sigma Chemical Co, St Louis, MO, USA). When a confluent monolayer of cells was formed (after 3–4 days), the cells were trypsinized. The Vero cells were then seeded into 4-well culture dishes (Nunc, Roskilde, Sweden), used for embryo co-culture when the monolayer is reached. BM1 medium (Ellios) was used for embryo co-culture. Two pronuclear (2PN) zygotes were placed in co-culture and maintained over the monolayer with no changes of medium until they reached the blastocyst stage, usually on days 5, 6, 7 or 8. Those blastocysts that were not transferred were frozen using glycerol (Sigma) as a cryoprotectant (Ménézo and Veiga, 1997). Only blastocysts showing normal morphology, having a well-defined ICM, trophoderm and adequate blastocoelic cavity, were used for replacement or freezing. Patients in their first IVF cycle, either conventional IVF or intracytoplasmic sperm injection (ICSI), were included in a randomized study to evaluate the usefulness of blastocyst transfer, in comparison with early stage (day 2) transfer. The mean age of the patients was 33.5 years, and the laboratory protocol was the same as that for the patients suffering from repeated implantation failure.

The sex and birthweight of babies born after blastocyst transfer were analysed in newborns from the Institut Rhônalpin (1991–1998) and Institut Dexeus (1995–1998). Blastocyst transfer was performed on both centres in patients suffering from previous implantation failure. They were compared.
**Table I.** Global results (August 1995–December 1997).
Values are given as mean values with range in parentheses, unless otherwise indicated

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cycles</td>
<td>384</td>
</tr>
<tr>
<td>Age (years)</td>
<td>35.8 (19–48)</td>
</tr>
<tr>
<td>Previous replacements</td>
<td>3.0 (0–14)</td>
</tr>
<tr>
<td>Embryos replaced</td>
<td>8.2 (0–36)</td>
</tr>
<tr>
<td>Infertility</td>
<td></td>
</tr>
<tr>
<td>tubal (%)</td>
<td>32</td>
</tr>
<tr>
<td>male (%)</td>
<td>33</td>
</tr>
<tr>
<td>endometriosis (%)</td>
<td>10</td>
</tr>
<tr>
<td>unknown (%)</td>
<td>11</td>
</tr>
<tr>
<td>failed AID (%)</td>
<td>5</td>
</tr>
<tr>
<td>Days of stimulation</td>
<td>10.1 (6–17)</td>
</tr>
<tr>
<td>No. of ampoules of gonadotrophins</td>
<td>31.1 (14–66)</td>
</tr>
<tr>
<td>No. of ampoules of oestradiol</td>
<td>1732 (339–3890)</td>
</tr>
<tr>
<td>No. of oocytes</td>
<td>12.7 (2–43)</td>
</tr>
<tr>
<td>No. of MII oocytes</td>
<td>10.7 (1–31)</td>
</tr>
</tbody>
</table>

AID = artificial insemination by donor; MII = metaphase II.

with babies born after spontaneous pregnancies in the Institut Dexeus in 1997 (a total of 927 boys and 889 girls).

The laser system used for assisted hatching and blastocyst biopsy for preimplantation genetic diagnosis (PGD) was a non-contact diode laser (1.48 μm Fertilase MTM, Lausanne, Switzerland). Assisted hatching was performed by laser zona pellucida drilling on mid-expanded blastocysts to increase implantation rates. Laser drilling was also used to perforate the zona pellucida of blastocysts to allow trophectoderm biopsy. After culture and herniation, the herniated portion was biopsied by laser and aspiration.

**Statistical analysis**

Student’s t-test and the $\chi^2$ test were used to compare quantitative and qualitative variables.

**Results**

The results are presented in Tables I and II. A total of 2868 zygotes were co-cultured and 1027 embryos reached the blastocyst stage, i.e. a blastocyst formation rate of 35.8%. Blastocysts ($n = 473$) were frozen and stored for a subsequent transfer in 43.7% of patients. The mean number of blastocysts replaced was 1.8 per patient and 95 pregnancies were obtained (24.7% pregnancy rate/patient, 31% pregnancy rate/transfer). The blastocyst implantation rate was 23.6%. Miscarriage occurred in 15 patients and ectopic pregnancy in three (15.7% abortion rate, 3.1% ectopic rate). The multiple pregnancy rate was 32.6% (26.3% twins and 6.3% triplets).

When patients were classified according to the number of oocytes retrieved, no significant differences were observed in the blastocyst and pregnancy rate/replacement between patients in whom $<5$, $6–15$ and $>15$ oocytes were obtained (36.4 and 23.7%, 26.6% and 38.7 and 35.5% respectively).

The blastocyst rate was significantly lower in patients where frozen donor spermatozoa were used, compared with conventional IVF or ICSI (21.7 and 38.8, 34.9% respectively; $P < 0.01$). The blastocyst and pregnancy rates were also significantly lower ($P < 0.001$ and $P < 0.05$ respectively) in patients aged $>40$ years (26.6% compared with 30.4, 38.7 and 35.5% for patients aged $<30$, 30–35, or 36–40 years respectively).

When looking at the characteristics of patients with at least one blastocyst, compared with those where no blastocyst was obtained, statistically significant differences were observed in the mean age (36.8 versus 35.3, $P < 0.01$) and in the number of oocytes (13.3 versus 9.8; $P < 0.01$) and mature oocytes (10.8 versus 7.4; $P < 0.01$). The pregnancy rate increased with the number of blastocysts replaced (16.5, 37.6, 37.5, and 75%...
Co-culture to improve implantation rates

Table III. Result of a randomized study on the effects of co-culture during the first cycle of in-vitro fertilization (IVF)

<table>
<thead>
<tr>
<th></th>
<th>Co-culture</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>32.9</td>
<td>34.2</td>
</tr>
<tr>
<td>No. of co-cultured zygotes</td>
<td>186</td>
<td>-</td>
</tr>
<tr>
<td>No. of blastocysts</td>
<td>60</td>
<td>-</td>
</tr>
<tr>
<td>Blastocyst rate (%)</td>
<td>48.4</td>
<td>-</td>
</tr>
<tr>
<td>No. (%) of replacements</td>
<td>18 (78.3)</td>
<td>24 (100)</td>
</tr>
<tr>
<td>Mean no. of blastocysts/embryo replaced</td>
<td>1.7</td>
<td>2.8</td>
</tr>
<tr>
<td>No. of pregnancies</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Pregnancy rate/patient (%)</td>
<td>30.4</td>
<td>41.7</td>
</tr>
<tr>
<td>Pregnancy rate/replacement</td>
<td>38.9</td>
<td>41.7</td>
</tr>
<tr>
<td>Implantation rate</td>
<td>21.8</td>
<td>27.9</td>
</tr>
</tbody>
</table>

No significant differences were found.

for one, two, three, or four blastocysts replaced respectively) as well as the multiple pregnancy rate (0, 33.9, 46.7 and 100% respectively).

Significantly higher pregnancy rates per replacement ($P < 0.05$) and implantation rates ($P < 0.05$) were found when the replacement was carried out on days 5 or 6, compared with days 7 or 8, with statistically significant differences (38.2 and 25.7%, 35.3 and 26.1% compared with 7.5 and 5.1%, 0 and 0%).

Statistically significant differences between pregnant and non-pregnant patients were also observed in terms of mean age (34.6 versus 35.8 years; $P < 0.05$), mean number of oocytes and mature oocytes (14.0 and 11.4 versus 12.3 and 9.8 respectively; $P < 0.05$) and blastocyst rate (46.2 and 31.9%; $P < 0.01$).

A randomized study was performed to assess the usefulness of the co-culture system in the first IVF cycle. No statistically significant differences were observed in the pregnancy rates per patient or per replacement. The implantation rate was 21.8% in the co-culture group, compared with 27.9% in day 2 replacement (differences not statistically significant; Table III).

The advantage of assisted hatching in blastocysts was assessed. Assisted hatching was performed using a laser. Even though no statistically significant differences were observed, higher pregnancy and implantation rates were obtained when laser drilled blastocysts were replaced, compared with non-drilled blastocysts (44.4 versus 23.8% and 30.6 versus 11.6%).

A total of 215 cycles were carried out with frozen–thawed blastocysts. The overall survival rate was 55.6% and the pregnancy rate per cycle, per replacement (1.5 blastocysts replaced) and implantation rates were 15.3, 22.7 and 17.8% respectively.

With regard to weight at birth and the sex ratio of babies ($n = 416$) born after blastocyst replacement using the Vero cell line system at Institut Rhônaip, Fondation Marcel Merieux (1991–1998) and Institut Universitari Dexeus (1995–1998), there were no statistically significant differences in the birth weight or babies weighing >4 kg. However, a significantly higher rate of boys, compared with girls, was observed, both overall and in the fresh replacement group when compared to the general population (58.9 versus 41.1% and 60.5 versus 39.5% respectively, $P < 0.01$) (Table IV).

The co-culture system may also be used to develop and optimize blastocyst biopsy conditions for PGD, using laser-assisted hatching in non-transferable blastocysts coming from 3PN, 1PN and multinucleated blastomeres, as reported previously (Veiga et al., 1997). Preliminary results show the safety and usefulness of the system. The efficiency of trophectoderm cell herniation was 72.2% (13/18) while the efficiency of biopsy and fixation were 76.9 and 80% respectively (10 out of 13 and eight out of 10).

Discussion

It has been shown that suboptimal embryo culture conditions may be partly responsible for the low rate of pregnancies after assisted reproductive techniques. Based on encouraging results obtained in farm animals by co-culturing embryos (Camous et al., 1984; Gandolfi and Moor, 1987; Eyestone and First, 1989) as a means of improving embryo development in vitro, co-culture of early preimplantation embryos with cellular monolayers and trophoblastic vesicles has been introduced in humans to overcome the developmental arrest that usually occurs at the time of genomic activation.

Although a number of studies have shown a beneficial effect of co-culture on the morphology
of human embryos (Bongso et al., 1993), there appears to be conflicting data on whether the viability of embryos is improved, in comparison with a routine embryo culture medium (Janny and Ménézo, 1994; Olivennes et al., 1994; Sakkas et al., 1994; Freitas et al., 1995). However, coculture which allows good quality human blastocyst seems particularly suitable in clinical practice for certain groups of patients, e.g. those with repeated failures of implantation. In the first clinical application of co-culture of embryos on Vero cells (Ménézo et al., 1992a), a 56% blastocyst formation rate and a pregnancy rate of 44% were obtained in a series of 62 patients with repeated failures of transfers. Further studies (Ménézo and Ben Khalifa, 1995) showed the advantages of co-culture over conventional embryo culture systems with a significant increase of the implantation rate per transferred embryo in patients with blastocyst replacement. In contrast, in a randomized study (Van Blerkom, 1993), in which embryos at the 2PN stage were cultured either in the presence or absence of Vero cells, improvement of the different parameters of embryonic development at the blastocyst stage was not observed. But in this experiment, the number of cells per blastocyst was, in both groups, unusually low.

According to our results, blastocyst development does not seem to be impaired in poor (<5 oocytes) or high responders (<15 oocytes) (36.4 and 36.9%), when compared with normal responders (5–15 oocytes) (34.8%). Blastocyst replacement is thus useful in patients with low and high numbers of oocytes.

Patients aged >40 years have a lower blastocyst rate, compared with younger patients, with statistically significant differences (Janny and Ménézo, 1996). This impairment, together with poor endometrial receptivity, may be responsible for the poor results obtained after IVF in this group of patients.

When looking at the characteristics of patients in whom no blastocyst is obtained (17.1% of the patients) it appears that the mean age is significantly higher and the number of oocytes and mature oocytes are significantly lower than patients with at least one blastocyst. Co-culture to the blastocyst stage has also a diagnostic approach in patients with previous replacements without pregnancy. The inability of the embryos to reach the blastocyst stage may be one of the reasons for the previous failures. After two cycles of co-culture without blastocysts, oocyte or sperm donation can be considered.

In a report on a randomized trial of blastocyst culture with sequential media (Gardner et al., 1998), the patients were mostly undergoing their first IVF cycle and showed a moderate to good response to gonadotrophin stimulation. The blastocyst rate was 46.5%. Pregnancy rates were 66% after transfer on day 3 and 71% after day 5 transfer, with more embryos being transferred on day 3. The implantation rate of blastocysts was significantly higher than that of cleavage-stage embryos transferred on day 3. The transfer of two blastocysts should, therefore, decrease multiple gestations. Blastocyst and pregnancy rates did not appear to be affected by age, even though the sample size was small (45 patients). ICSI had no apparent effect on either the pregnancy or implantation rates, since the rates were similar in the 15 patients undergoing ICSI and the 30 patients undergoing IVF.

Pregnancy and implantation rates decreased dramatically when blastocysts were replaced on days 7 or 8 when compared with replacements carried out on days 5 or 6. The replacement was done as
soon as the blastocyst stage was reached. It was very important to assess blastocyst morphology and only structures with a defined ICM, trophoderm and blastocoelic cavity were considered to be real blastocysts. Endometrial–embryonic synchrony is lost from day 7 and thus, since June 1997, replacements have only been performed on days 5 and 6. When the blastocyst stage is reached after day 6, blastocysts are frozen and replaced in a subsequent artificial cycle.

Patients pregnant after blastocyst replacement were significantly younger than non-pregnant and also more oocytes and mature oocytes were obtained in the pregnant group. The blastocyst rate per patient was also significantly higher when pregnancy is achieved (46.2 versus 31.9%).

Patients in their first IVF cycle showed a higher blastocyst rate than the one observed in patients with previous implantation failures (48.4 versus 35.8%). No advantage was found with respect to pregnancy rate in replacing blastocysts compared with early cleavage stages in our randomized study. Blastocyst transfer is thus better used for difficult cases, e.g. patients with previous implantation failures (Olivennes et al., 1994; Bavister and Boatman, 1997).

Assisted hatching with the use of laser seems to improve blastocysts implantation rate even though larger series are needed to confirm the results. Jones and co-workers reported that the removal of the zona pellucida of blastocysts prior to replacement significantly improves implantation rates (Jones et al., 1998).

The results obtained with blastocyst freezing using a simplified technique (Ménézo and Veiga, 1997) give better survival rates than those obtained with early cleavage stages. Larger reports demonstrate higher survival rates (Ménézo et al., 1992b; Ménézo and Benkhalifa, 1995) and similar pregnancy and implantation rates. Pregnancy and implantation rates are higher than the ones obtained when freezing and thawing of cleaving stage embryos. Blastocyst freezing enables the cryopreservation of previously selected embryos and gives higher expectation of pregnancy to the patients. It also avoids cryostorage of large numbers of embryos with low implantation possibilities. Our freezing programme is actually performed at the blastocyst stage. A ‘take home baby rate’ of 10% per frozen blastocyst can be expected (Ménézo and Veiga, 1997).

The birthweight of children arising from blastocyst replacement was analysed in 416 babies, by combining the results from Institut Dexeus (89 babies) and Institut Rhônalpin (327 babies). No differences were observed in the mean birth weight from ‘blastocyst babies’ compared to ‘early cleavage babies’. The proportion of babies weighing >4 kg was not higher after blastocyst transfer (Ménézo et al., 1998). The phenomenon of large offspring ‘large calf syndrome’, described in the cow and which is related to early embryo culture conditions (Behboodi et al., 1994), does not seem to be observed here. Even though the sample is large, more data on birthweight are still needed. Moreover, it will be interesting to evaluate the results obtained with the use of sequential media.

When looking at the sex ratio of the newborn infants obtained (data from Institut Dexeus and Institut Rhônalpin) it appears that there are significantly more boys (58.9% for boys, 41.1% for girls, \( P < 0.01 \)). It has been suggested that male embryos grow faster in in-vitro conditions. This has been described in bovine embryos with more males in the fast growing embryos and more females in the slow growing ones (Avery et al., 1992). Accelerated cleavage is also evident in mice and cattle. In the rat and in the human, a qualitative sex difference is observed before testicular differentiation and this may increase the probability of the gonad reaching the threshold for testis development as developmental delay can result in ovarian differentiation (Mittwoch, 1993). It has also been demonstrated that in-vitro conditions support the development of more mouse male embryos to the blastocyst stage compared with female embryos. Embryonic energy metabolism during the preimplantation stage can be a candidate for this selection (Peippo and Bredbacka, 1995). In-vitro matured/IVF ovine male embryos have a fast development pattern, in comparison with females (Bernardi and Delouis, 1996). In the human, it has been demonstrated that the number of cells in male embryos is significantly higher on day 2 and this difference is maintained up to the blastocyst stage. Differences in embryo metabolism
are also reported (Ray et al., 1995). It has also been suggested that sex selection may be carried out inadvertently in IVF programmes due to the selection of fast cleaving embryos for replacement (Tarin et al., 1995). Other reports do not confirm these data (Ng et al., 1995). As blastocyst replacement is performed in both centres as soon as the blastocyst stage is reached, we are probably selecting more male blastocysts (that reach this stage before female embryos) for replacement and thus altering the sex ratio. Data from experiences with sequential media will be of great value to confirm the hypothesis.

Preliminary data on laser blastocyst biopsy for PGD show high rates of herniation, biopsy and fixation efficiency (72.2, 76.9 and 80% respectively). Even though technical improvements have enabled successful clinical application, it seems that blastocyst biopsy is a useful tool for PGD as the number of cells for diagnosis is increased thus improving the reliability of the procedure. Diagnostic possibilities may be enhanced by splitting the biopsy in small fragments.

**Conclusion**

Culture of embryos to the blastocyst stage is useful for patients with previous implantation failures, for blastocyst freezing and for PGD (Bavister and Boatman, 1997). No improvement is observed in patients in their first IVF cycle. By replacing one or two blastocysts on days 5 or 6, the multiple pregnancy rate will be significantly reduced without decreasing pregnancy rates. As previously suggested (Gardner, 1999), high order multiple gestations should be eliminated without decreasing pregnancy rates, after the transfer of just two blastocysts. Assisted hatching with laser on blastocysts prior to replacement seems to improve implantation rates although larger series are needed to draw conclusions. Blastocyst replacement alters the sex ratio by enhancing the birth of more boys compared to girls. The use of co-culture has assisted the understanding of blastocyst development and morphology. The use of sequential media should confirm all the information collected from co-culture.

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