Live births in poor prognosis IVF patients using a novel non-contact human endometrial co-culture system

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Abstract

Patients with repeated implantation failures or poor embryo quality may benefit from embryo culture using the co-culture technique; growth factors secreted by co-culture cells may act as survival factors. Autologous endometrial co-culture has been suggested as a safe alternative to animal cells for co-culture of human embryos. However, the technique is fairly labour intensive and its effectiveness can vary from patient-to-patient. This study presents clinical outcome data on a novel non-contact co-culture system using a human endometrial cell line rather than autologous tissue. Embryos from 316 poor prognosis patients with repeated IVF failures, previous cycles with poor embryo quality or advanced maternal age were cultured in Transwell chambers with a monolayer of endometrial cells. The clinical pregnancy rate in patients less than 39 years of age was 53% and for patients aged between 39 and 42 years it was 33%. To date, 76 patients have delivered 111 healthy infants with no congenital anomalies and 18 pregnancies are ongoing. This is the first report on the potential benefits of a non-contact co-culture system in the IVF laboratory. This study shows that an established human endometrial cell line can be used to obtain the benefits of co-culture without the potential disadvantages associated with using autologous endometrial tissue.

Keywords: co-culture, cytokines, embryo transfer, endometrium, growth factor, IVF

Introduction

Over the past decade, researchers have focused on trying to optimize in-vitro culture systems by adjusting media formulations to better mimic the female reproductive tract. A major stumbling block to performing day-5 transfers for all patients has been continuing concern over potential retardation of embryo development during extended time in culture in an environment devoid of growth factors. Numerous data point to growth factor involvement in embryonic genome activation, blastocyst differentiation and modulation of apoptosis (Schultz and Heyner, 1993; Collins et al., 1994; Chegini, 1996; Brison and Schultz, 1997; O’Neill 1997; Byrne et al., 2002).

In-vitro exposure of embryos to growth factors by co-culture on a feeder cell monolayer was one of the earliest methodologies used to increase the blastulation rate of human embryos and to aid embryonic development in poor prognosis patients. Clinical IVF trials have been conducted using co-culture with both bovine and human oviductal tissue (Wiemer et al., 1989, 1993, 1998; Bongso et al., 1990, 1994) as well as human endometrium (Simon et al., 1999; Rubio et al., 2000; Spandorfer et al., 2001, 2004, 2006; Mercader et al., 2003). Co-culture using primary tissue is a fairly labour intensive process requiring tissue retrieval, enzymatic digestion, isolation and propagation of desired cell types.

An alternative to the culture of primary tissues has been the use
of an established cell line. The Vero cell line, originally derived from African Green Monkey kidneys, has been successfully used for co-culture in fertility clinics worldwide (Menezo et al., 1990, 1992; Schillaci et al., 1994; Veiga et al., 1999). This epithelial cell line is easily passaged and can be purchased from the World Health Organization or the American Type Culture Collection. Recent concerns over exposure of human embryos to animal cells have, however, made the Vero cell co-culture system less tenable for use in clinical IVF laboratories.

A more attractive option for co-culture in the clinical setting may be the use of an established human endometrial cell line. A co-culture system that utilizes a novel human endometrial cell line has been previously described (Desai et al., 1994; Desai and Goldfarb, 1996). This permanent cell line has embryotrophic properties and compares favourably to both Vero cells and human oviductal cells as a co-culture system. Blastocysts derived from endometrial cell co-culture show enhanced blastomere counts (Desai et al., 2003) even when compared with embryos cultivated in commercially available sequential culture medium systems such as the G2.3 series (VitroLife, Sweden).

The primary objective of this report is to present the clinical outcome data, including live births, obtained using this novel endometrial cell co-culture system for poor prognosis patients. A non-contact co-culture system for human IVF is also described for the first time.

Materials and methods

Patients

This study was conducted at the Cleveland Clinic Fertility Centre in Beachwood, Ohio. Patients gave informed consent for the IVF treatment and laboratory procedures. Embryos from all poor prognosis patients in the IVF programme were cultured in the novel co-culture system. A total of 316 poor prognosis patients were treated between January 2004 and March 2007.

Patients were categorized as having poor prognosis based on the following criteria: two or more previous implantation failures with good quality embryos; previous failed cycle(s) with poor embryo quality (low cell number/high fragmentation); or advanced maternal age (≥39 years). No other patient selection or exclusion criteria were used. Patients were subsequently stratified into three groups according to their age at the time of embryo transfer: group 1 <39 years (n = 103); group 2 39–42 years old (n = 173); and group 3 ≥42 years old (n = 40).

Ovarian stimulation/embryo transfer

Ovulation induction was carried out after down-regulation with leuprolide acetate (Lupron; TAP, Pharmaceuticals, Lake Forest, IL) and stimulation with recombinant FSH (Follistim; Organon or Gonal F; Serono). FSH was initiated at a dose of 100 IU on day 3 and increased to 300 IU on day 5 of the menstrual cycle. Human chorionic gonadotropin (HCG) was administered when at least two follicles were 14 mm in diameter. Embryos were transferred 3 days after the oocyte retrieval under ultrasound guidance using a Wallace Sure View catheter.

Oocyte fertilization/culture

Oocytes were fertilized by intracytoplasmic sperm injection 3–4 h after retrieval. Injected oocytes were cultured in microwells of human tubal fluid medium (LifeGlobal, Guilford, CT) supplemented with 10 mg/mL of human serum albumin (Cooper Surgical Inc., Trumball, CT) under an oil overlay. Fertilization was checked 18–20 h after ICSI. Normally fertilized zygotes were separated and placed in co-culture wells.

Co-culture on endometrial cells

The source and the preparation technique for the endometrial cell line have been previously described (Desai et al., 1994). The cell line was passaged twice a week. For co-culture, human endometrial cells were seeded into the outer well of a Costar Transwell dish (Fisher Scientific, Houston, TX) at a concentration of either 15,000 or 30,000 cells per well (Figure 1). Endometrial cell monolayers were utilized for co-culture when they were approximately 50% confluent. The monolayer and the membrane insert were rinsed twice with culture medium before placing embryos in the wells. Global Blastocyst Medium (LifeGlobal, Guilford, CT) supplemented with 10% Synthetic Serum Substitute (SSS; Irvine) was used for the co-culture treatment. Culture medium in Transwells was refreshed daily. All cultures were incubated at 37°C with 5.5% CO₂. Embryo development was monitored daily.

Embryo selection

Embryo selection for day-3 transfer was based solely on morphological parameters. Embryos were graded on the basis of cell number, regularity of blastomeres, good blastomere expansion, fragmentation level and signs of embryonic compaction. Transfer number was based primarily on patient age. Patients less than 39 years generally received two or three embryos, depending on embryo quality and the patient’s acceptability of selective reduction in case of high order multiple pregnancy. Patients 39 years and older usually had three or four embryos transferred.

Surplus embryos not transferred or frozen on day 3 were kept in co-culture and frozen at the blastocyst stage. Embryo cleavage, compaction, morulation and blastulation were monitored from day 3 to day 6 for these embryos.

Outcome measures

Pregnancy testing was performed 15 days after embryo transfer. Clinical pregnancy was confirmed by the presence of a fetal heart on ultrasonic examination at 6–8 weeks of pregnancy. The implantation rate was derived from the number of fetal hearts divided by the total number of embryos transferred. Multiple pregnancy, live births and congenital malformations were monitored.
Results

Co-culture with this novel endometrial cell line was easily introduced into the IVF laboratory. The Transwell chamber with its 0.4 µm membrane allowed fluid exchange but prevented direct contact between endometrial cells and human embryos during the co-culture interval. To optimize human embryo development, a commercially available human blastocyst culture medium was used. Global Blastocyst Medium was selected since it can be used as a single one-step system for culture of human embryos from zygote to blastocyst and can also support proliferation of endometrial cells (unpublished data). Medium exchanges were easily performed from the outer well of the chamber. Daily half change of medium was an effective means of maintaining the pH in the desired range of 7.2–7.4 and preventing osmolarity shifts during culture at 37ºC without an oil overlay.

Clinical outcome data with co-culture in poor prognosis patients is presented in Table 1. The data are stratified according to patient age. The youngest (<39 years) group comprised patients with previous implantation failure. Endometrial co-culture appeared to have a positive effect on embryo development. Embryos in the transfer cohort exhibited less than 20% fragmentation, good blastomere expansion, and some signs of compactation on day 3, an early positive indicator of genomic activation. A clinical pregnancy rate of 53% was achieved. Despite previous failures, the implantation rate per embryo transferred in this group was high (30%); an indirect reflection of the potential positive effect of the co-culture treatment.

Table 2 depicts a further subgrouping of patients with previous implantation failure and their subsequent outcomes. Co-culture treatment was selectively applied to patients <39 years with only a single previous IVF failure in instances where overall embryo quality was exceptionally poor with high levels of fragmentation. All of these patients had low cell number, poor morphology and/or >25% fragmentation at transfer on their prior IVF attempt. In this group, 11 patients had failed treatment in other IVF programmes, and their records indicated compromised embryo quality. The rationale for the use of co-culture was an attempt to ‘rescue’ their embryos and so improve the chance of success.

The impact of endometrial co-culture on patients in the older age group (39–42 years) with a mean age of 40 ± 1.0 years was difficult to discern. Although a high percentage of patients had positive pregnancy tests (56%), the clinical pregnancy rate was only 33%. It is likely that embryonic aneuploidy associated with advanced maternal age may significantly impact on the ultimate clinical pregnancy outcome and live birth rate in this group of patients, as well as in the oldest (>42 years) age group. The potential benefit of co-culture in the older patients might simply be to mediate the support of embryonic development and genomic activation through growth factors secreted by the cell monolayer.

It was observed that surplus embryos from these older patients progressed to the blastocyst stage in the co-culture environment. The percentage of blastocysts formed was not different from that observed in the under 39 age group. Figure 2 depicts the morphological stage of embryos during the culture interval. Extended culture of surplus embryos not transferred or frozen on day 3 provided an opportunity to make daily observations on the developmental pattern in endometrial cell co-cultured embryos. By day 5 of culture, 63% of surplus embryos had compacted or reached the morula stage and 25% had reached the blastocyst stage. By day 6 of culture, 42% of surplus embryos co-cultured with endometrial cells had blastulated.

The final outcome measure assessed was live birth rate following embryo co-culture with human endometrial cells. A total of 119 (37.7%) of the 316 patients who received embryo
Table 1. IVF outcome after co-culture of embryos with a human endometrial cell line.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patient age group (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;39</td>
</tr>
<tr>
<td>No. of embryo transfers</td>
<td>103</td>
</tr>
<tr>
<td>Mean age (years)</td>
<td>34.3 ± 4.6</td>
</tr>
<tr>
<td>Mean no. of cycles</td>
<td>3.0 ± 1.7</td>
</tr>
<tr>
<td>Mean no. of oocytes retrieved</td>
<td>13.1 ± 6.6</td>
</tr>
<tr>
<td>Mean no. of oocytes fertilized</td>
<td>7.9 ± 4.1</td>
</tr>
<tr>
<td>Mean no. of embryos transferred</td>
<td>2.8 ± 0.7</td>
</tr>
<tr>
<td>No. of cycles with positive HCG (%)</td>
<td>65/103 (65)</td>
</tr>
<tr>
<td>Clinical pregnancy rate (%)</td>
<td>54/103 (53)</td>
</tr>
<tr>
<td>Implantation rate (%)</td>
<td>89/290 (31)</td>
</tr>
<tr>
<td>Deliveries (pending)</td>
<td>41 (7)</td>
</tr>
<tr>
<td>Live births to date</td>
<td>70</td>
</tr>
<tr>
<td>Singleton births</td>
<td>17</td>
</tr>
<tr>
<td>Twins</td>
<td>19</td>
</tr>
<tr>
<td>Triplets</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 2. Clinical pregnancy rate (%) in patients with previous implantation failure.

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>One failed cycle</th>
<th>2–3 failed cycles</th>
<th>4 or more failed cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;39</td>
<td>50 (23/46)</td>
<td>58 (23/40)</td>
<td>47 (8/17)</td>
</tr>
<tr>
<td>≥39</td>
<td>26 (14/54)</td>
<td>20 (9/46)</td>
<td>32 (9/28)</td>
</tr>
</tbody>
</table>

*Includes patients with one or more failed cycles with poor embryo quality in another IVF clinic.

Figure 2. Human embryos not utilized on day 3 were kept in the Transwell co-culture system until day 6. The percentage of embryos at each cell stage is depicted for each day of culture.

Discussion

Despite the tremendous improvements in blastocyst culture media, the lack of growth factors in culture milieu may still hamper overall embryonic development in vitro (Roberts, 2005; Sjoblom et al., 2005; Urman and Balaban, 2005). The in-vivo milieu in the oviduct and later in the uterus is far more complex than any commercially available medium. The multitude of growth factors and maternally derived biofactors found in oviductal and uterine fluid may profoundly affect development and implantation processes. Significant efforts have been made to properly characterize endometrial cells and study their functions in vitro (Stavreus-Evers et al., 2003). The ability of embryos to adapt and grow in vitro, even under nutritional stress, often masks our understanding of the optimal culture conditions. For example, while embryos can blastulate in the absence of granulocyte–macrophage colony stimulating factor (GM-CSF), a cytokine secreted by the endometrium, this results in sub-optimal fetal growth and subfunctional placentation (Sjoblom et al., 2005). Moreover, it has been shown that other growth factors such as leukaemia inhibitory factor (LIF) and interleukin (IL)-1 system are associated with endometrial receptivity (Bulletti et al., 2005).
Communication or ‘cross-talk’ between the endometrium and the embryos is another emerging area of interest. Endometrial cells can regulate expression of cytokines such as IL-1 by human embryos (De los Santos et al., 1996). Conversely, data suggest that the presence of embryos can increase mRNA expression for IGF (insulin-like growth factor) binding protein in endometrial cells (Lai et al., 1996; Soong et al., 1998). Localized embryo-mediated stimulation of specific factors at the site of implantation in the uterus may be important.

Creating a more physiological environment for embryonic development using human endometrial cell co-culture may be a useful strategy for aiding embryo development in poor prognosis patients, and this needs further exploration. Co-culture cells could potentially provide bioactive factors and ‘cross-talk’ that is absent in IVF culture media alone. Growth factors supplied by co-culture cells may aid suboptimal embryos by reducing environmental stress, decreasing potential sublethal damage to cells that may trigger apoptotic pathways and interfere with normal inner cell mass and trophectoderm development. The co-culture cells may also facilitate removal of free radicals and other toxic metabolites from the culture environment of the embryo (Ouhibi et al., 1990; Bongso et al., 1995).

Autologous endometrial co-culture has been used successfully in clinical IVF to improve embryo quality and treat poor prognosis patients (reviewed in Birkenfeld and Navot, 1991; Barmat et al., 1999; Simon et al., 1999; Mercader et al., 2003; Spandorfer et al., 2004, 2006). However, this approach requires the patient to undergo endometrial biopsy in anticipation of their cycle. Moreover, much variability between patients exists since each patient’s ‘co-culture system’ is a different mix of stromal and epithelial cells derived from their own uterine lining. In contrast, utilization of a permanent human endometrial cell line for co-culture may be a more effective technology, allowing standardization of the co-culture treatment across patients.

The human endometrial cell line described in this paper was characterized as primarily epithelial and documented to express LIF, platelet-derived growth factor (PDGF) and IL-6, similar to the Vero cell line (Desai et al., 1994; Desai and Goldfarb, 1996). This cell line is easily passaged and cryopreserved, making its application to assisted reproductive procedures more attractive than the use of autologous tissue.

This report presents the first clinical data on IVF and live births using an established human endometrial cell line. The Transwell system combined with this novel human endometrial cell line provides a unique non-contact system for human embryo coculture. One of the advantages of this design is that the embryos can benefit from embryotrophic factors secreted by the coculture cells but still remain totally isolated, thus abolishing the risk of inadvertent transfer of endometrial cells into the uterus along with the embryo. The Transwell system has been used very successfully with other cell types (Germeraad et al., 1994; Villars et al., 1996; Murdoch et al., 2007). The membrane is non-toxic and translucent, allowing good visualization during the growth interval. Medium is easily exchanged from the outer well without disturbing the embryos.

The initial clinical findings are very promising and suggest that this culture system may benefit specific groups of poor prognosis patients. The clinical pregnancy rate of 53% in the youngest (<39 years of age) group compares favourably to outcomes after co-culture with autologous human endometrial cells. Spandorfer and colleagues in their largest series obtained a 42% clinical pregnancy rate in patients with repeated failed IVF cycles (Spandorfer et al., 2004). They also noted that the timing of endometrial biopsy impacted strongly on pregnancy outcome (Spandorfer et al., 2002). Co-culture with endometrial cells harvested early in the cycle resulted in a lower pregnancy rate than endometrial cell monolayers derived from mid-luteal phase endometrium (19 versus 45%, respectively). Other investigators using autologous endometrial co-culture for IVF patients have published clinical pregnancy outcomes ranging from 20–34% (Jayot et al., 1995; Simon et al., 1999; Rubio et al., 2000; Mercader et al., 2003).

Endometrial cell co-culture as a clinical treatment option is ongoing in the authors’ IVF programme. Growth factors secreted into the culture milieu by the feeder cells may help to promote the development of suboptimal embryos and improve outcomes in poor prognosis patients with repeated IVF failures. A further study of the cell line and its secretions is underway in an effort to better understand the impact of co-culture on human embryonic development. Ultimately, a prospective trial comparing embryonic performance in blastocyst medium with and without a feeder layer will be necessary to validate the efficacy of this co-culture system for treatment of poor prognosis patients.

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