Human Fallopian tube epithelial cell co-culture increases fertilization rates in male factor infertility but not in tubal or unexplained infertility

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Abstract

In order to investigate the effect of human Fallopian tube epithelial cell co-culture on fertilization and cleavage rates in tubal, male and unexplained infertility, oocytes collected from 91 patients were randomized to wells containing Fallopian tube epithelial cell monolayers or conventional culture medium, and inseminated with spermatozoa. Fertilization and cleavage were assessed at 18 and 52 h, respectively. Co-culture significantly increased the fertilization rates over the control values in male infertility (41.67 versus 23.43%, P = 0.00005), but not in tubal infertility (69.33 versus 67.93%) or unexplained infertility (65.93 versus 54.36%). Cleavage rates were not different in co-culture and conventional in-vitro fertilization systems in any of the infertility subgroups. The number of blastomeres was significantly higher in the co-culture group on the day of embryo transfer (3.63 ± 1.12 versus 3.04 ± 1.26, P < 0.001). Pregnancy rates were similar in all infertility subgroups. There was no significant association between the number of co-cultured embryos transferred and the pregnancy, abortion and multiple pregnancy rates. It was concluded that human Fallopian tube epithelial cell co-culture clearly improves fertilization rates in male infertility but not in tubal or unexplained infertility. Improved fertilization rates in co-culture may be due to positive effect of co-culture on impaired sperm function.

Key words: co-culture/Fallopian tube epithelial cells/in-vitro fertilization

Introduction

There is increasing evidence that co-culture systems in assisted reproduction have beneficial effects (Bongso et al., 1991b, 1993a,b; Bongso and Fong, 1993). Co-culture systems increase fertilization rates (Bongso et al., 1991a), embryo quality (Wiemer et al., 1989a; Bongso et al., 1989, 1992; Freeman et al., 1995), embryo development and blastocyst formation rates (Bongso et al., 1989, 1992; Wiemer et al., 1989b, 1993; Ménézo et al., 1990, 1992; Plachot et al., 1993) and pregnancy rates (Wiemer et al., 1989a, 1989b, 1993; Ménézo et al., 1990; Bongso et al., 1992; Jayot et al., 1995). In these studies human Fallopian tube epithelial cells (Bongso et al., 1989, 1991a, 1992), human endometrial cells (Jayot et al., 1995), human granulosa cells (Plachot et al., 1993; Freeman et al., 1995), bovine uterine and oviductal epithelial cells (Wiemer et al., 1989a, 1989b, 1993) or established cell lines (e.g. Vero cells) (Ménézo et al., 1990, 1992) were used as supportive cells.

In all but one of these studies co-culture was performed using the pronuclear stage zygote after fertilization in conventional culture media; the effects on early embryo quality and development, blastocyst formation rates and/or pregnancy rates were examined (Bongso et al., 1989, 1992; Wiemer et al., 1989a, 1989b, 1993; Ménézo et al., 1990, 1992; Plachot et al., 1993; Freeman et al., 1995). A single study by Bongso et al. (1991a) examined fertilization rates in co-culture systems. In a group of patients with male factor infertility and tubal disease, they reported improved fertilization rates when the gametes were co-cultured with epithelial cells before attachment to the culture well. Fertilization rates in the two subgroups were not specified.

Freeman et al. (1995) reported similar clinical pregnancy and implantation rates in tubal, endometriosis, male factor, unexplained and ‘other’ infertility groups using a granulosa cell co-culture system. The blastocyst formation rates of untransferred embryos were lower in the unexplained infertility group than in the tubal, endometriosis and ‘other’ groups.

In the present study, we have investigated the effect of Fallopian tube epithelial cell co-culture on fertilization and cleavage rates in a group of patients with tubal, male or unexplained infertility.

Materials and methods

Patients and ovarian stimulation regime

This study was carried out in the In Vitro Fertilisation–Embryo Transfer Centre, International Hospital, Istanbul, Turkey. The hospital ethics committee approved inclusion of those patients who had more than eight oocytes at egg recovery and permission was given for the use of ~30% of eggs for the co-culture study, in order to minimize any potential disturbance to the patients’ standard treatment. Ninety-one patients who had more than eight oocytes at egg recovery was included, following informed consent. All patients and their partners had complete investigation including laparoscopy, at least two semen analyses, endometrial biopsy and assessment of male and female antisperm antibodies. In all, 37 (40.6%) patients had male factor infertility, 35 (38.5%) had tubal disease and 19 (20.9%) unexplained infertility. Semen analyses were performed as recommended by the World Health Organization (WHO, 1992). Patients were included in the male infertility group when the sperm concentration was <10 x10⁶/ml, percentage of motile spermatozoa <40% and abnormal形态.
forms >70%. Tubal disease group patients had bilateral tubal blockage or severe pelvic adhesions. In cases of unexplained infertility, all investigations were normal. All couples with male or unexplained infertility had at least three cycles of intrauterine insemination before being admitted to the in-vitro fertilization (IVF) programme.

The treatment protocol was identical in all cases. Pituitary down-regulation was achieved using long protocol buserelin 1000 µg/day, starting on cycle day 1–3 or 19–21. This was followed by ovarian stimulation with human menopausal gonadotrophin 150–225 IU daily. Follicular development was monitored with ultrasound scanning and serum eestradiol measurements. Ovulation was triggered by the administration of 10 000 IU human chorionic gonadotrophins after at least three leading follicles reached a mean diameter of 16–18 mm and serum oestradiol was >1000 pg/l. Ultrasound-guided egg recovery was performed under sedation or general anaesthesia. A weighted randomization method (Altman, 1991) was used to allocate ~30% of the eggs to the co-culture study and the remaining 70% to conventional IVF.

Fallopian tube epithelial cell cultures
Fallopian tube epithelial cell cultures were prepared as described previously (Kervancioglu et al., 1994b). Fallopian tubes were obtained from two premenopausal women, aged 36 and 39 years, who gave informed consent for removal of their Fallopian tubes during a total abdominal hysterectomy for uterine fibroids. The patients were screened for human immunodeficiency virus (HIV) and hepatitis B pre-operatively. After excision, the mucosal folds of the Fallopian tubes were dissected out, cut into 1 mm³ pieces and placed in flasks containing 1 ml of minimum essential medium in Earle’s salts with L-glutamine (MEM–Earle’s; Gibco, Paisley, UK) supplemented with streptomycin (Evans Medical, Surrey, UK) (50 µg/ml), penicillin (Glaxo, Middlesex, UK) (100 IU/ml) and 40% fetal bovine serum (FBS, Sigma, Poole, UK). The primary cultures were then incubated at 37°C in an atmosphere of 5% CO₂, 95% air and 95% humidity.
Fallopian tube epithelial cell co-culture

Figure 5. Fertilization rates in the co-culture and conventional in-vitro fertilization (IVF) culture systems in patients with tubal, male or unexplained infertility. The difference between fertilization rates in co-culture and conventional IVF was not significant in the tubal and unexplained infertility groups, but was significant in the male factor infertility group (*P = 0.00005).

Figure 6. Cleavage rates in co-culture and conventional in-vitro fertilization culture systems in patients with tubal, male or unexplained infertility. The difference was not significant in any of the groups.

After 3 days a further 4 ml of culture medium containing 10% FBS was added. New epithelial cells were observed after 3–4 days of primary culture and epithelial patches were seen after 7–10 days. The cells which had become adherent to the primary culture dishes were washed with calcium- and magnesium-free Hanks’ balanced salt solution (Gibco), then trypsinized with 3 ml of 0.05% trypsin and 0.02% EDTA (Gibco) and subcultured into 4 ml of culture medium containing 15% FBS. The cells were frozen at confluence (4–5 days), using the same trypsinization technique. The cell pellet after trypsinization was resuspended in MEM–Earle’s containing 20% FBS at a concentration of 10^6 cells/ml. Dimethyl sulphoxide (Sigma) was added dropwise at a final concentration of 10% (v/v), agitating the cells to seed each well was 1 ml. These dishes were used for co-culture after the epithelial cells had reached confluence (Figure 1). Two wells of each dish were left for conventional culture as control. The epithelial origin of the cells was confirmed by immunofluorescence and transmission electron microscopy (TEM, Philips EM400, Eindhoven, Holland). Immunofluorescence showed that at least 80% of the cells contained cytokeratin (Figure 2) and TEM revealed the presence of reticulated Golgi complexes, abundant granular endoplasmic reticulum, variably shaped mitochondria, electron dense granules, lipid droplets and intracellular junctions (Figure 3). All cell cultures were routinely screened for bacteria, fungi and viruses.

Co-culture

After confluence (usually on day 2 or 3) the cells were washed with MEM–Earle’s followed by three 30 min washes with MEM–Earle’s supplemented with streptomycin, penicillin and 10% patient’s serum. Patient’s serum was replaced with Albuminaz® (LKB Biotek, Milton Keynes, UK) for those patients with unexplained infertility. Control wells contained the same culture medium without the cells.

The oocytes were allocated to the co-culture or conventional IVF wells using a weighted randomization method (Altman, 1991), and were matured for 6–8 h at 37°C in an atmosphere of 5% CO2/95% air and 95% humidity. Sibling oocytes were used as controls (conventional IVF) to the co-culture group within the same patient. It was assumed that random allocation of oocytes to treatment provided similar grade oocytes in the co-culture and control groups. The spermatozoa were prepared on discontinuous Percoll (Sigma) as described by Aitken et al. (1993). Oocytes were inseminated with 75 000 spermatozoa per oocyte when there was no male infertility and 150 000 spermatozoa per oocyte in cases of male infertility. However, the same numbers of spermatozoa per oocyte were used for both conventional IVF and co-culture within the same patient. Fertilization was checked 18 h later. Fertilized oocytes with two pronuclei were transferred to new wells containing the same culture medium with or without the epithelial cell monolayers. The oocytes were assessed for cleavage 24 h later (Figure 4).

Embryo transfer (one to four embryos depending on number available for transfer) was performed 52–54 h after the oocyte recovery. Embryos with the best morphology were chosen for transfer and patients received a combination of embryos developed in co-culture and conventional IVF. Spare embryos were discarded, as embryo freezing was illegal in Turkey.

Results

A total of 1185 oocytes were included in this study; 397 (33.5%) were inseminated in the co-culture system and 788 (66.5%) in the conventional IVF. The ages (mean ± SD) of the female/male patients were 33.2 ± 3.4/38.8 ± 5.9 years (tubal disease), 31.9 ± 4.9/35.4 ± 4.4 years (male infertility) and 33.2 ± 4.9/36.5 ± 4.3 years (unexplained infertility). The mean ages of these groups were comparable (Student’s t-test). The numbers of eggs collected at oocyte recovery were 440, 459 and 286 for the tubal factor, male and unexplained infertility groups respectively (12.6, 12.6 and 15.0 per patient respectively). Of these oocytes, 150 (34.1%), 156 (34.0%) and 91 (31.8%) were used for the co-culture in the tubal factor, male and unexplained infertility groups respectively.

The overall fertilization rates in the co-culture and conventional culture systems were 57.68% (329/573) and 47.46% (374/788) respectively (F = 0.00089, χ²-test). The overall
The fertilization rates were not significantly different ($\chi^2$-test) between the co-culture and conventional culture systems respectively in the tubal (69.33 versus 67.93%) or unexplained (65.93 versus 54.36%) infertility groups, whereas these numbers were significantly different in the male infertility group (41.67 versus 23.43%, $P = 0.00005$) (Figure 5).

Once the oocytes were fertilized the cleavage rates did not differ in the co-culture and conventional culture systems in any of these groups (84.62 versus 79.19% for the tubal infertility group, 81.54 versus 77.46% for the male infertility group and 90.0 versus 79.2% for the unexplained infertility group) (Figure 6). The mean number of blastomeres on the day of embryo transfer was significantly higher in the co-culture group (3.63 ± 1.12) than in the conventional culture (3.04 ± 1.26) ($P < 0.001$, Student’s $t$-test). The clinical pregnancy rates were 32.4, 29.4 and 28.1% in the tubal, unexplained and male infertility groups respectively and these were not significantly different ($\chi^2$-test). The pregnancy, abortion and multiple pregnancy rates in relation to the number of co-cultured embryos transferred are given in Table I. Although there was a tendency towards higher pregnancy rates with increased number of co-cultured embryos transferred, this did not reach statistical significance ($\chi^2$-test). Abortion and miscarriage rates were not significantly related to the number of co-cultured embryos transferred.

### Table I. Pregnancy rates per embryo transfer, abortion and multiple pregnancy rates per clinical pregnancy in relation to the number of co-cultured embryos transferred

<table>
<thead>
<tr>
<th>No. of co-cultured embryos transferred</th>
<th>Pregnancy rates</th>
<th>Abortion rates</th>
<th>Multiple pregnancy rates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tubal $(n = 35)$</td>
<td>Unexp. $(n = 19)$</td>
<td>Male $(n = 37)$</td>
</tr>
<tr>
<td>0</td>
<td>4/14 (28.6)</td>
<td>2/9 (22.2)</td>
<td>2/12 (16.7)</td>
</tr>
<tr>
<td>1</td>
<td>4/13 (30.8)</td>
<td>1/3 (33.3)</td>
<td>3/9 (33.3)</td>
</tr>
<tr>
<td>2</td>
<td>2/5 (40.0)</td>
<td>2/3 (66.6)</td>
<td>3/7 (42.8)</td>
</tr>
<tr>
<td>3</td>
<td>1/2 (50.0)</td>
<td>0/2 (0)</td>
<td>2/5 (40.0)</td>
</tr>
<tr>
<td>Overall</td>
<td>11/34 (32.4)</td>
<td>5/17 (29.4)</td>
<td>10/33 (28.1)</td>
</tr>
</tbody>
</table>

$P$ values reflect the statistical comparison within each column in relation to the number of co-cultured embryos transferred. Unexp. = unexplained infertility.

Discussion

In addition to their beneficial effects on early embryo development, co-culture systems can improve sperm movement characteristics (Guerin et al., 1991; Wetzels et al., 1991; Bongso et al., 1993b; Pearlstone et al., 1993), induce sperm capacitation (Kervancioglu et al., 1994a) and increase sperm survival (Kervancioglu et al., 1994a; Mansour et al., 1995). Therefore, higher fertilization rates would be expected in culture systems using supportive cell layers. However, there is only one study in the literature using a co-culture system to investigate fertilization rates in the human (Bongso et al., 1991a). In this study, gametes were co-cultured with epithelial cells before they were attached to the floor of the wells. This was because sperm binding to attached epithelial cell monolayers was observed which appeared to restrict the movement of spermatozoa.

Sperm binding to epithelial cells in co-culture systems is a well-known phenomenon (Pacey et al., 1995) and the significance of this observation is not clear. In animal studies, it has been shown that contact between spermatozoa and oviduct epithelial cells is beneficial for sperm survival both in vivo (Smith and Yanagimachi, 1990) and in vitro (Pellard et al., 1991) and sperm capacitation may be stimulated by this interaction (Ellington et al., 1991). Furthermore, improved fertilization rates can be achieved in mice by co-culture with human Fallopian tube epithelium monolayers (Goldberg et al., 1991). Therefore, the interaction between spermatozoa and epithelial cells may be beneficial rather than a disadvantage. For this reason, we decided to use cell monolayers rather than unattached epithelial cells to study the effect of co-culture on fertilization rates. To our knowledge, this study is the first to investigate fertilization rates using epithelial cell monolayers in a human IVF and embryo transfer programme.

There is no published comparison of the effects of co-culture on normozoospermic samples and oligoasthenozoospermic samples, although several studies confirmed that both normozoospermic (Kervancioglu et al., 1994a) and asthenozoospermic (Chen et al., 1994) samples may benefit from co-culture. Similarly, most of the co-culture studies did not compare the pregnancy rates in subgroups of patients with different aetiology of infertility. Freeman et al. (1995) reported similar pregnancy and implantation rates in various subgroups of infertility, while the blastocyst formation rate of untransferred embryos was lower in the unexplained infertility group. However, there was no control group.

We have now studied, for the first time, the fertilization and cleavage rates in different infertility subgroups using conventional IVF as control. The results show that the positive effect of co-culture on fertilization rates was seen mainly with...
suboptimal semen samples; fertilization rates in the male infertility subgroup were much superior in the co-culture system (Figure 5, $P = 0.00005$). While no beneficial effect was seen in patients with tubal infertility, there appeared to be some improvement in the fertilization rates in the unexplained infertility group although this did not reach statistical significance (Figure 5, $P = 0.064731$). This suggests that there may be a subgroup of patients in the unexplained infertility group with impaired sperm function which cannot be detected by routine semen analysis.

Co-culture may also have positive effects on oocytes. The oviduct may enhance various functions of the zona pellucida in the hamster (Yang and Yanagimachi, 1989) and also secretes polypeptides incorporated into the oocyte membrane in sheep (Gandolfi et al., 1989). Therefore, an improvement in the outcome of IVF may be expected in all infertility subgroups. However, in the present study the fertilization rates for tubal factor and unexplained infertility patients did not show a significant difference between co-culture and conventional IVF culture systems. It is possible that co-culture may improve the embryo development at a later stage through its positive effects on oocytes. Further studies are needed to investigate the effects of co-culture on oocytes.

This study showed no improvement in the cleavage rates in the overall group or in any subgroup. In fact, there is no evidence in the literature for a positive effect of co-culture on oocytes. Further studies are needed to investigate the effects of co-culture on oocytes.

In conclusion, this is the first study comparing the effects of co-culture on human zygote development. Although the numbers in each group were small. Another interesting point is that the pregnancy rates were similar in the male infertility group to those in the tubal and unexplained infertility groups. Although lower pregnancy rates are normally expected in male infertility, this was not the case in this study. This improvement may be due to the effect of co-culture but the direct evidence is lacking.

In contrast to previous studies using Fallopian tube epithelial cells, frozen–thawed epithelial cells were used here to allow screening of the donors of the Fallopian tubes for HIV and hepatitis B 6 months after the operation, in addition to the pre-operative screening and routine screening for bacteria, fungi and viruses. The use of fresh cells from another patient carries the risk of disease transmission even if the patient is screened pre-operatively, because of the latent phases of these viruses. Freezing may have some adverse effects on cell functions, and the secretory activity of these cells may be impaired (Saridogan et al., 1997). Since the beneficial effects of co-culture are probably due to certain secretory proteins as well as to reduction of oxygen tension or neutralization of toxic reactive oxygen species (Thibodeaux and Godke 1992), the use of frozen–thawed cells may have certain implications and disadvantages. Nevertheless, co-culture with these cells had a clear positive effect on fertilization rates.

The overall fertilization rate in this study was relatively low. However, >40% of the patients had male infertility and the expected low fertilization rate in this group contributed to the overall low fertilization rate (Figure 5). Low fertilization rate in male infertility was one of the major problems in assisted reproduction until the development of microinjection techniques. Currently, high fertilization rates can be achieved with intracytoplasmic sperm injection, and co-cultures may not be needed. However, microinjection requires expensive set-up procedures and trained personnel, while co-cultures can be established in routine IVF laboratories. Furthermore, co-cultures can still be used after microinjection for the enhancement of early embryo development.

In conclusion, this is the first study comparing the effects of co-culture in various subgroups of infertile patients. Our results suggest that co-culture improves fertilization rates in cases of male infertility, while it has no effect on fertilization rates in tubal and unexplained infertility. Improved fertilization rates in co-culture may be due to a positive effect of Fallopian tube epithelial cells on impaired sperm function, a biological effect which needs further investigation.

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References


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