Development of hatching blastocysts from immature human oocytes following in-vitro maturation and fertilization using a co-culture system

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Recently, in-vitro maturation (IVM) of immature human oocytes recovered from non-stimulated follicles has been applied in the treatment of infertility. However, in previous reports, very few embryos cultured in conventional medium have reached the expanded blastocyst stage following in-vitro maturation and fertilization (IVM/IVF). The objective of this study was to investigate whether the developmental competence of human embryos following IVM/IVF could be enhanced by the use of a human ampullary cell co-culture system. Immature human oocytes were aspirated from small follicles at Caesarean section and then cultured in medium containing human menopausal gonadotrophin for 36 to 48 h, followed by insemination. Zygotes were randomly cultured either in conventional culture medium alone or in the co-culture system. Of 48 embryos cultured in conventional medium alone, all arrested at the 2–16-cell stage on day 3 after insemination. Of 46 embryos cultured in the co-culture system, 26 embryos (56.5%) arrested at the 2–16-cell stage. Six embryos (13%) developed to the morula stage. Fourteen embryos (30.4%) developed to expanded blastocysts and two blastocysts were hatching on day 7 after insemination. We conclude that co-culture significantly enhances the development of blastocysts in embryos resulting from IVM/IVF.
Key words: ampullary cell/blastocyst/co-culture/human/in-vitro maturation

Introduction

Immature human oocytes in non-atretic pre-ovulatory follicles, like those in other mammals, can achieve maturation *in vitro* from germinal vesicle breakdown to the metaphase II arrest. In-vitro maturation of mammalian oocytes was first reported in rabbits by Pincus and Enzmann (1935). In-vitro maturation (IVM) and in-vitro fertilization (IVF) of immature oocytes recovered from non-stimulated ovaries have been successfully performed in farm animals, resulting in pregnancy and birth (Goto *et al.*, 1988; Fukuda *et al.*, 1990). In humans, in-vitro maturation studies were first performed by Pincus and Saunders (1939). During the 1960s, Edwards further demonstrated that

in-vitro matured human oocytes could be fertilized *in vitro* (Edwards *et al.*, 1969). Recently, the use of immature human eggs for the initiation of pregnancy has been achieved (Cha *et al.*, 1991; Trounson *et al.*, 1994), although the developmental competence of embryos resulting from in-vitro matured and fertilized (IVM/IVF) human oocytes has rarely been reported. Trounson *et al.* (1994) observed that IVM/IVF embryos from polycystic ovary syndrome (PCOS) patients can be cultured for up to 68 h after insemination. In a case report, one early blastocyst was produced 110 h after intracytoplasmic sperm injection from IVM oocytes of a PCOS patient. The embryo was transferred to the patient's uterus and resulted in the birth of a baby (Barnes *et al.*, 1995).

Meanwhile, progress in the technique of co-culture has recently led to the production of blastocysts following IVM/IVF in farm animals (Goto *et al.*, 1988; Eyestone and First, 1989). In humans, some studies have reported that the rate of blastocyst formation in co-cultured embryos was significantly greater than in those cultured in conventional medium alone. Co-cultured human embryos also had higher pregnancy and implantation rates (Ménézo *et al.*, 1990; Bongso *et al.*, 1991; Wiemer *et al.*, 1993).

The purpose of this study was to investigate if the developmental competence of embryos following IVM/IVF could be enhanced using a co-culture system. To the best of our knowledge, this is the first study reporting the effect of co-culture on the development of blastocysts resulting from immature human oocytes matured and fertilized *in vitro*.

Materials and methods

### Collection of immature human oocytes

After counselling, informed patients consented to donate immature oocytes for research. The ages of patients ranged from 20 to 35 years. The gestational ages of pregnancy ranged from 37 to 41 weeks. The oocytes were collected at Caesarean section and the study was conducted between March 1996 and December 1996. All immature oocytes were aspirated at the time of Caesarean section from small follicles with a 20- or 21-gauge needle connected to a 2.5-ml syringe filled with 1 ml of HEPES-buffered human tubal fluid (HTF) culture medium. The diameters of follicles ranged from 2 to 6 mm. All visible follicles were aspirated. Immediately after recovery, the compact cumulus cells of some oocytes were partially removed using glass pipettes to examine for the existence of a germinal vesicle (Figures 1, 2). All immature oocytes, including partially denuded oocytes, were cultured for maturation, except those that were already degenerate at the time of oocyte recovery.

Oocyte maturation and fertilization in vitro

All immature oocytes were cultured with their attached compact surrounding cumulus cells in maturation medium which contained
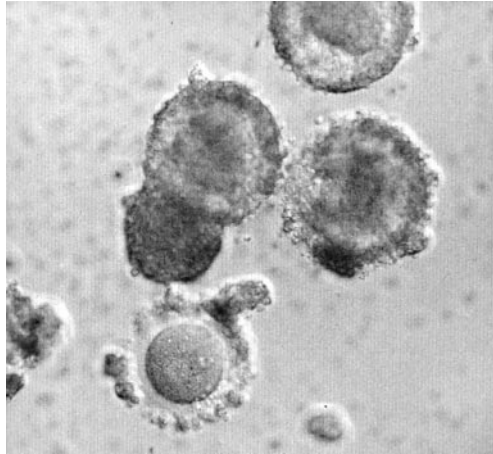
Figure 1. A group of immature oocytes surrounded by compact cumulus cells retrieved from small follicles at the time of Caesarean section. Original magnification ×100.
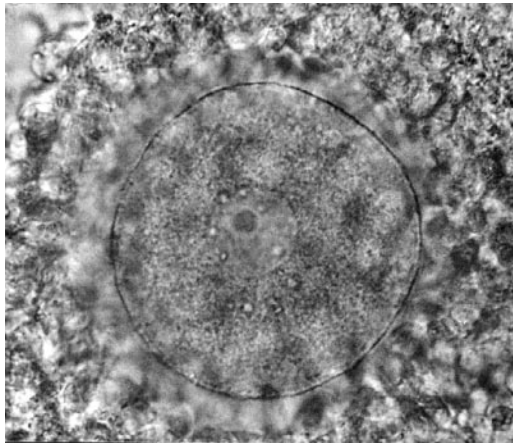
Figure 2. A germinal vesicle at the centre of an immature oocyte. Original magnification ×400.

HTF medium supplemented with 150 mIU/ml human menopausal gonadotrophin (Pergonal, Serono, Rome, Italy), 10% fetal cord serum (FCS) and 1 μg/ml oestradiol (Sigma, St. Louis, MO, USA). One to three immature oocytes were placed in a drop of 30 μl maturation medium under mineral oil in a Petri dish and cultured at 37°C in an atmosphere of 5% CO_2 in air. After 36 to 48 h of incubation, the maturation status was evaluated. Oocytes with a visible polar body (mature metaphase II, Figure 3) were then inseminated. The inseminating spermatozoa were donated from normal fertile men after semen analysis and were prepared by the swim-up technique. The mature metaphase II oocytes were transferred to drops of 25 μl HTF medium supplemented with 10% FCS and 50 000 to 100 000 motile sperm/ml under mineral oil in a Petri dish.

Fetal cord serum was collected from the delivery room in our hospital after the baby was born and centrifuged immediately at 700 *g* for 10 min. After centrifugation, the plasma was removed and transferred to another centrifuge tube at room temperature to allow clot formation. After removal of the clot, the serum was heat inactivated at 56°C for 30 min and stored at –70°C until required. In this study, the same batch of FCS was used in all the HTF culture media.
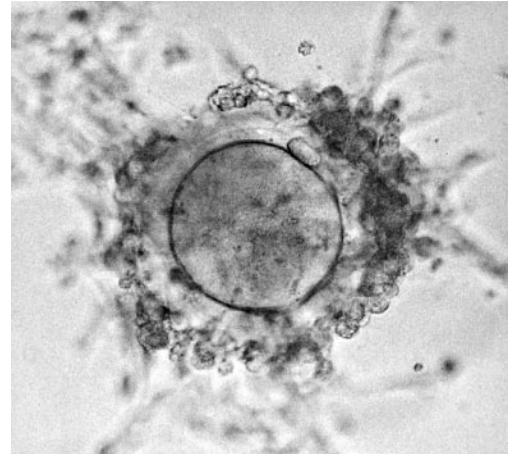
Figure 3. Mature oocyte demonstrating the first polar body extruded after maturation *in vitro*. Original magnification ×200.

Preparation and establishment of the human ampullary cell co-culture system

The human ampullary cells used in this study were obtained from patients under 40 years old who underwent salpingo-oophorectomy or hysterectomy due to non-malignant diseases. Immediately after the Fallopian tubes were excised, small pieces of endosalpinx tissue (1 to 3 mm^2) were teased or cut away from the ampullary portion of the Fallopian tube using forceps and scissors. These small pieces of endosalpinx tissue were washed several times in Hanks' balanced salt solution (HBSS) (Gibco BRL, Grand Island, NY, USA) at 37°C to remove blood. The pieces of endosalpinx tissue were then transferred to a centrifuge tube containing 5 ml 0.05% trypsin-EDTA (cat. no. 25300-054, Gibco BRL) and incubated at 37°C in an atmosphere of 5% CO_2 in air for 30 to 50 min to separate and slough the epithelial cell clusters. After incubation, 3 to 4 ml of turbid supernatant containing epithelial cells was removed and transferred to another centrifuge tube containing 10 ml Chang's medium (cat. no. T102-000, Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% fetal bovine serum (FBS) (cat. no. 1210378, JRH Biosciences, Lenexa, KS, USA). Following centrifugation at 250 *g* for 10 min, the supernatant was discarded. The remaining cell pellet was resuspended in 2 ml Chang's medium with 10% FBS and transferred to a 50-ml tissue culture flask (Greiner) containing 5 ml of Chang's medium with 10% FBS. This flask was incubated at 37°C in an atmosphere of 5% CO_2 in air. The culture medium was changed every 3 days.

It usually took 3 days for the cells to attach and 5 to 7 days to grow to confluent monolayers. After this primary culture monolayer was prepared, primary culture epithelial cells were ready to be detached from the flask for subculturing. This was accomplished by decanting the Chang's medium in the tissue culture flask and washing the flask with 5 ml HBSS. This solution was discarded and replaced with 1 to 2 ml of 0.05% trypsin-EDTA. The flask with 1 to 2 ml of 0.05% trypsin-EDTA was then incubated at 37°C with an atmosphere of 5% CO_2 in air for 5 min to detach the cells. The medium containing detached cells was mixed with 10 ml Chang's medium with 10% FBS and was transferred to a centrifuge tube and centrifuged at 250 *g* for 10 min. The supernatant was aspirated and discarded to remove the trypsin and the cell pellet was resuspended in 5 ml Chang's medium with 10% FBS. To set up the subculture, 1 ml of this cell suspension was transferred to a new flask containing 5 ml Chang's medium with 10% FBS.

The procedures for the subculture were the same as for the primary culture. Once monolayers of the subculture were formed, further

passages of the subculture were performed in the same way. In this study, only the first three passages of ampullary epithelial cell monolayers were used to co-culture the embryos.

Embryo culture and human ampullary cell co-culture

About 16 to 20 h after insemination, the oocytes were examined. The presence of two pronuclei and two polar bodies were used as the criteria of normal fertilization. Oocytes with three pronuclei were not used in this study. The normal fertilized oocytes obtained from each patient were randomly divided into two groups: the co-culture group and the control group. In the control group, fertilized oocytes were cultured in drops of 25 µl HTF medium with 10% FCS serum under mineral oil (Sigma) in Petri dishes.

In the co-culture group, the fertilized oocytes were co-cultured on human ampullary epithelial cells. One day before insemination, the subcultured ampullary monolayer cells were washed twice with HBSS containing 10% FBS. Then, 2 ml of 0.05% trypsin-EDTA was added to the flask to detach the subcultured monolayer cells. After 5 min incubation at 37°C in 5% CO_2 in air, the medium containing detached cells was transferred to a centrifuge tube containing 10 ml Chang's medium supplemented with 10% FBS and centrifuged at 250 *g* for 10 min. The supernatant was discarded and the cell pellet was resuspended in Chang's medium supplemented with 10% FBS. After this wash procedure, 0.5 ml of the cell suspension (concentration of 2×10^5 cells/ml) was transferred and seeded in 4-well dishes which were used for the embryo co-culture. Therefore, about 1×10^5 ampullary cells were seeded in each well of the 4-well dishes. These 4-well dishes containing epithelial cells were incubated at 37°C in 5% CO_2 in air. Two days later, the Chang's medium was removed and the 4-well dishes containing seeded cells were washed twice with HBSS. HTF medium with 10% FCS was used to wash the dish for a third time, after which 0.5 ml HTF medium with 10% FCS was added to each well and covered with mineral oil. After 6 h incubation at 37 °C in 5% CO_2 in air, about two to three fertilized oocytes were placed in each well of the 4-well dishes for embryo co-culture. The embryos were transferred every 2 days to the new 4-well dishes containing newly seeded subcultured ampullary epithelial cells which were prepared using the procedure aforementioned.

The cleavage of zygotes and embryo development were evaluated to the blastocyst stage over the next few days. In this study, according to Dokras' blastocyst grading system (Dokras *et al.*, 1993), only fully expanded blastocysts containing a distinct inner cell mass and trophectoderm layer (grade 1 and grade 2 blastocysts) were recorded as blastocysts.

Statistical analysis was carried out using the χ^2 test. A *P*-value of < 0.05 was considered significant.

Results

A total of 268 immature oocytes were aspirated from small follicles of 51 patients at the time of Caesarean section. The mean number of immature oocytes retrieved from each patient was 5.3 (range 1 to 15). The immature oocytes were all surrounded by compact cumulus cells, which made it difficult to measure the diameter of the immature oocytes precisely, since the cumulus cells covered the outer margin of the zona pellucida. Therefore, we did not measure the diameters of all the immature oocytes, although we did measure some healthy-looking immature oocytes with a visible outer margin of the zona pellucida. These oocytes had the same diameter (including zona pellucida but not including surrounding granulosa cells) as mature oocytes retrieved from patients in a clinical IVF
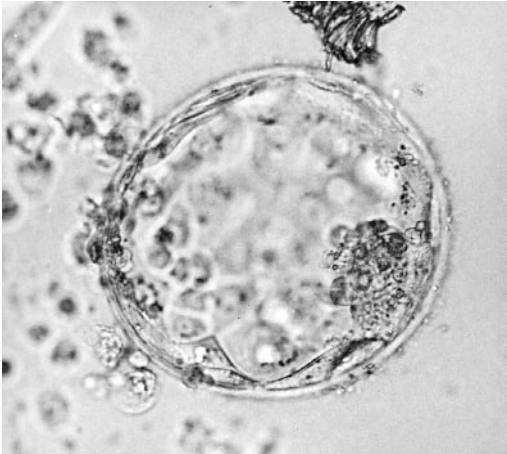
Figure 4. A co-cultured embryo following in-vitro maturation and fertilization reaching the expanded blastocyst stage on day 6 after insemination. Original magnification ×400.

Table I. Comparison of the developmental capacity of embryos following in-vitro maturation and fertilization in the control and the co-culture groups

Stage of embryos	Control group	Co-culture group
No. of two pronucleate embryos	54	53
Total no. of cleaved embryos	48	46
2–16-cell	48 (100%)	26 (56.5%)
Morula	0	6 (13%)
Blastocyst	0[a]	14 (30.4%)[a]

[a]Values were significantly different from each other ($P < 0.001$) using χ^2 test.

programme. Most of the small immature oocytes (<110 µm) were degenerate and were not used in this study. Of 268 immature oocytes, 39 (14.6%) were already degenerate at the time of recovery. The remaining 229 oocytes were incubated for maturation *in vitro*. Forty-eight hours later, extrusion of the first polar body was evident in 154 oocytes (67.2%). After insemination, 107 out of 154 oocytes (69.5%) were confirmed to be normally fertilized (two pronuclei). Five of the 154 oocytes (3.2%) were found to have three pronuclei.

Of the 107 normally fertilized oocytes, 54 were randomly placed in the control group and 53 in the co-culture group. In the control group, 48 out of 54 fertilized oocytes (88.9%) cleaved. All of these 48 embryos arrested at the 2–16-cell stage on day 3 after insemination (Table I). According to our routine IVF protocol, we transfer embryos on day 2 following insemination (about 42 to 46 h following insemination), at which time most are at the 2–16-cell stage. In this study, the development rates of embryos following IVM were comparable to those of regular clinical IVF embryos in the first 48 h following insemination.

In the co-culture group, 46 of the 53 fertilized oocytes (86.8%) cleaved. Twenty-six of these 46 embryos (56.5%) arrested at the 2–16-cell stage on day 3 after insemination. Six embryos (13%) developed to morulae. Fourteen embryos (30.4%) developed to expanded blastocysts on day 6 after insemination (Figure 4). Of these, two were hatching on day 7 after insemination (Figure 5) (Table I). Embryos in the co-culture group had significantly higher rates of development to

Figure 5. Co-cultured blastocyst almost hatched from its zona pellucida on day 7 after insemination. Original magnification ×200.

the blastocyst stage (14/53) than the control group (0/54; $P < 0.001$).

Discussion

In vivo, immature human oocytes (germinal vesicle stage) resume meiosis, the germinal vesicle breaks down and the first polar body is extruded only shortly after the influence of the luteinizing hormone (LH) surge (Edwards, 1965a; Eppig, 1993). Without the LH surge, immature oocytes *in vivo* cannot reach metaphase II and ovulation. Normally the pre-ovulatory LH surge occurs in the middle of the menstrual cycle (on day 12 to 14 of a normal 28-day cycle). However, when immature oocytes were isolated from small antral follicles and cultured *in vitro* with follicle stimulating hormone (FSH) and LH in the culture medium, they could resume meiosis within 48 to 54 h (Trounson *et al.*, 1994). Two possible mechanisms might account for this. Firstly, follicular fluid in small antral follicles possesses maturation inhibitor activities (Channing *et al.*, 1983). Immature oocytes liberated from their small antral follicular environment are exposed to a reduction of maturation inhibitory signal. The immature oocytes may undergo spontaneous maturation *in vitro* at a rate similar to that in the ovary after stimulation by LH in many mammalian species and humans (Edwards, 1965b). Secondly, gonadotrophins or growth factors added to the culture medium may induce maturation *in vitro*. This mechanism involves the production of a positive stimulatory factor by the cumulus cells that acts on the oocyte to trigger maturation. This mechanism bypasses the negative influence of the meiosis-arresting pathway (Downs *et al.*, 1988). Based on the results of this study and a previous report (Trounson *et al.,* 1994), high concentrations of FSH and LH in the culture medium may act like the LH surge and induce resumption of meiosis in immature oocytes *in vitro* within 48 h.

In this study, all the immature oocytes were recovered from patients at the time of Caesarean section. The mean number of immature oocytes recovered from each patient was 5.3, which is lower than the mean number recovered from excised ovarian specimens (11.7) (Cha *et al.*, 1991) and from PCOS patients (15.3) (Trounson *et al.*, 1994). In the past, very few reports concerning follicular development in the human ovary

during late pregnancy have been reported. Although follicular maturation and ovulation are interrupted, small antral follicles continue to grow during late pregnancy (Govan *et al.*, 1970). Under high levels of human chorionic gonadotrophin during late pregnancy, small antral follicles grow only to about 4 to 5 mm in size before atresia occurs. The follicular fluid of atretic follicles appears to be absorbed, and ultimately the antrum disappears and a solid mass of theca cells is formed. Our findings that all follicles had diameters smaller than 6 mm during Caesarean section are consistent with previous reports (Govan, 1970; Starup and Visfeldt, 1974).

When compared with other studies (Cha *et al.*, 1991; Trounson *et al.*, 1994), the low recovery rate of immature oocytes in this study may be due to a high rate of degeneration of small antral follicles in late pregnancy and may also be explained by the fact that only follicles at the ovarian surface can be aspirated. Some follicles located beneath the ovarian surface were not visible and, thus, not aspirated during Caesarean section. In this study, the maturation rate of oocytes after 48 h of culture was 67.2% which is lower than the maturation rate of 81% reported by Trounson *et al.* (1994). However, in this study the fertilization rate (69.5%) and the cleavage rate (86.8%) were higher than the fertilization rate (34%) and the cleavage rate (56%) reported by Trounson. We have no clear explanation for this fact. The differences in the microenvironment of the different sources of immature oocytes may be one of the possible reasons.

Very few studies have investigated the hormonal environment of small antral follicles during late gestation. Westergaard *et al.* (1985) found that the concentrations of steroids (androstenedione, testosterone and oestradiol) in antral follicles during late pregnancy were approximately the same as in follicles of similar size from non-pregnant women, with the exception of progesterone. The progesterone levels in follicular fluid from pregnant women were significantly higher than from non-pregnant women (Westergaard *et al.*, 1985). Eden *et al.* (1990) reported that follicles of patients with PCOS contained significantly higher levels of androstenedione and lower oestradiol than normal control women. However, Westergaard (1992) demonstrated that concentrations of progesterone, androstenedione and oestradiol do not significantly differ between healthy follicles and atretic follicles with diameters between 2 and 6 mm. When the diameters of follicles exceeded 6 mm, concentrations of oestradiol increased significantly in healthy follicles compared with those in atretic ones (Westergaard, 1992). Westergaard *et al.* (1985) suggested that small antral follicles (<6 mm diameter) may develop and function independently of cyclic gonadotrophin secretion. Therefore, the mechanisms regulating the growth and atresia of small follicles (<6 mm diameter) are similar during pregnancy and the menstrual cycle. Previous studies have suggested that nonsteroidal growth factors produced in the ovary may regulate the development of antral follicles smaller than 6 mm in diameter (Westergaard *et al.*, 1990). Eden *et al.* (1990) reported that follicular fluid from patients with PCOS contained significantly higher concentrations of insulin-like growth factor-I than follicular fluid from patients without PCOS matched for follicular fluid volume. Westergaard *et al.* (1990) studied the

levels of epidermal growth factor (EGF) in follicular fluid aspirated from small follicles with diameters from 1 to 6 mm in women undergoing Caesarean section at term. He found a significant inverse correlation between concentrations of EGF and follicular size. He suggested that EGF is specific to small antral follicles and that EGF inhibits FSH-induced aromatase activity, thus protecting the small follicles from an untimely effect of FSH. When the diameters of follicles grow to 5 to 6 mm, EGF levels drop to a level which cannot inhibit FSH. However, FSH levels remain very low during late pregnancy. When the follicles grow to 5 to 6 mm in diameter during late pregnancy, they will become atresic due to lack of FSH stimulation.

In this study, almost all the immature oocytes were aspirated from small follicles with a diameter less than 6 mm. Thus, these immature oocytes could be rescued from atresia after being isolated and cultured in medium supplemented with FSH and LH. In patients with PCOS, Franks *et al.* (1988) also found high concentrations of EGF in follicular fluid from small follicles. In a report by Trounson *et al.* (1994), immature oocytes were retrieved from follicles with diameters from 2 to 10 mm using vaginal ultrasound, thus, some immature oocytes were obtained from follicles larger than 6 mm. Previous reports showed that women with PCOS undergoing IVF had significantly lower fertilization and cleavage rates than patients with tubal infertility. This difference was attributed to the poor quality of oocytes retrieved from patients with PCOS (Dor *et al.*, 1990; Tarlatzis *et al.*, 1997).

Because various authors have used different sources of immature oocytes and have retrieved different percentages of healthy oocytes for maturation *in vitro*, it is difficult to explain exactly why we had higher fertilization and cleavage rates in our study than those in other reports. The higher fertilization and cleavage rates in our study may be due to the higher percentage of healthy immature oocytes that we used. However, more studies are needed to elucidate this question and to determine the best source of healthy immature oocytes.

In humans, previous studies have reported that pregnancies can be produced from IVM/IVF of oocytes recovered from non-stimulated ovaries (Cha *et al.*, 1991; Trounson *et al.*, 1994). However, the conventional culture system used in previous reports was inadequate for the culture of human embryos following IVM/IVF to the blastocyst stage. In our preliminary experiments, human embryos resulting from IVM/IVF immature oocytes stopped developing at the 2–16-cell stage on day 3 after insemination, when cultured in the conventional culture system. The total length of time to culture immature human oocytes to the expanded blastocysts in the co-culture group ranged from 7 to 9 days in this study. In contrast, embryos in the control group stopped developing on day 3 after insemination. Based on the results of the present study and our preliminary experiments, there is still no ideal culture medium which can adequately support human immature oocytes developing to expanded blastocysts when using the conventional culture system. Up until now, to our knowledge, only one case report has demonstrated that an early blastocyst can be obtained from immature oocytes recovered from a PCOS patient using a conventional culture system (Barnes *et al.*, 1995).

With conventional human IVF, there is no apparent 'block' in embryo development, although the percentage reaching the blastocyst stage is low. Many previous studies have demonstrated that a co-culture system significantly enhances the percentage of embryos developing to the blastocyst stage (Ménézo *et al.*, 1990; Bongso *et al.*, 1991; Wiemer *et al.*, 1991). Meanwhile, in animal studies, the combination of IVM/IVF with subsequent co-culture on oviduct cells produced a high percentage of blastocysts (Fukuda *et al.*, 1990; Kim *et al.*, 1990; Wiemer *et al.*, 1991). In previous reports on clinical human IVF, co-culture has been used to improve embryo development and implantation rates (Plachot, 1996). Two hypotheses have been suggested to explain how co-culture cells provide their beneficial effects for embryo development (Bongso *et al.*, 1991). Firstly, co-culture cells may secrete embryotrophic factors such as growth factors, cytokines, or oviduct-specific glycoproteins. Leukaemia inhibitory factor has been demonstrated in Vero cell monolayers (Papaxanthos-Roche *et al.*, 1994). Transforming growth factor-alpha, EGF, and insulin-like growth factor have been demonstrated in human Fallopian tubes (Pfeifer and Chegini, 1994; Smotrich *et al.*, 1996). Secondly, the co-culture system could remove some toxic compounds from the culture medium. In this study, among the 54 zygotes in the control group, no embryos developed to the morula or blastocyst stage. In contrast, 13% of embryos developed to the morula stage and 30.4% reached the expanded blastocyst stage when the embryos following IVM/IVF were co-cultured with ampullary epithelial cells. This study shows that co-culture can overcome the problem of in-vitro developmental arrest at the 2–16-cell stage on day 3 after insemination.

In humans, to the best our knowledge, this is the first report of co-culturing IVM/IVF embryos with human oviduct epithelial cells. Furthermore, two expanded blastocysts achieved hatching on day 7 after insemination in this study. To our knowledge, this is also the first report to demonstrate that immature human oocytes recovered from non-stimulated ovaries can develop to hatching blastocysts by the use of a co-culture system.

Based on the results of this study, we suggest that co-culture can be added to IVM programmes to enhance blastocyst development and hatching. Although it is still difficult to evaluate the true efficacy of co-culture and its mechanism of action (Plachot, 1996), the results of this study indicate clearly that human immature oocytes retrieved from small follicles at Caesarean section may develop to the hatched blastocyst stage when co-cultured with human ampullary cells. We also tried other alternatives to the co-culture which included M3 medium (Medicult, Denmark) and α-minimum essential medium (Desai *et al.*, 1997) in place of HTF medium for small numbers of IVM/IVF embryos. In our limited experience up until now, co-culture still gives the best chance of enhancing the development of blastocyst resulting from IVM/IVF embryos (unpublished data).

In the future, the use of immature oocytes from Caesarean sections may not only contribute to the oocyte donation

programme, but may also be used for pregnant women using IVF procedures who desire another pregnancy but who do not have spare frozen embryos. We also concur with Bavister's opinion that the inadequacies of current culture medium allow co-culture to appear beneficial (Bavister, 1992). Further studies are required to develop the ideal culture medium to replace the co-culture system and adequately support human immature oocytes to reach the expanded blastocyst stage.

Acknowledgements

References

Barnes, F. L., Crombie, A., Gardner, D.K. et al. (1995) Blastocyst development and birth after in-vitro maturation of human primary oocytes, intracytoplasmic sperm injection and assisted hatching. Hum. Reprod., **10**, 3243–3247.

Bavister, B.D. (1992) Co-culture for embryo development: is it really necessary? Hum Reprod., **7**, 1339–1441.

Bongso, A., Ng, S.C., Fong, C. Y. et al. (1991) Cocultures: a new lead in embryo quality improvement for assisted reproduction. Fertil. Steril., **56**, 179–191.

Cha, K.Y., Koo, J. J., Ko, J.J. et al. (1991) Pregnancy after in-vitro fertilization of human follicular oocytes collected from non-stimulated cycles, their culture in vitro and their transfer in a donor oocyte program. Fertil. Steril., **55**, 109–113.

Channing, C.P., Liu, C.Q., Jones, G.S. et al. (1983) Decline of follicular oocyte maturation inhibitor coincident with maturation and achievement of fertilizability of oocytes recovered at midcycle of gonadotropin-treated women. Proc. Natl. Acad. Sci. USA, **80**, 4184–4188.

Desai, N., Kinzer, D., Loeb B. et al. (1997) Use of synthetic serum substitute and α-minimum essential medium for the extended culture of human embryos to the blastocyst stage. Hum. Reprod., **12**, 328–335.

Dokras, A., Sargent, I.L., Barlow, D.H. et al. (1993) Human blastocyst grading: an indicator of developmental potential? Hum. Reprod., **8**, 2119–2127.

Dor J., Shulman A., Levran D., Ben-Rafael Z., Rudak E. et al. (1990) The treatment of patients with polycystic ovary syndrome by in-vitro fertilization and embryo transfer: a comparison of results with those of patients with tubal infertility. Hum. Reprod., **5**, 816–818.

Downs, S.M., Daniel, S.A.J, and Eppig, J.J. (1988) Induction of maturation in cumulus cell-enclosed mouse oocytes by follicle stimulation hormone and epidermal growth factor: evidence for a positive stimulus of somatic cell origin. J. Exp. Zool., **245**, 86–96.

Eden, J.A, Jones, J, Carter, G.D. et al. (1990) Follicular fluid concentrations of insulin-like growth factor 1, epidermal growth factor, transforming growth factor-alpha and sex-steroids in volume matched normal and polycystic human follicles. Clin. Endocrinol., **32**, 395–405.

Edwards, R. G. (1965a) Maturation in vitro of human ovarian oocytes. Lancet, **ii**, 926–929.

Edwards, R.G. (1965b) Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey, and human ovarian oocytes. Nature, **208**, 349–351.

Edwards, R.G., Bavister, B.D., Steptoe, P.C. (1969) Early stages of fertilization in vitro of human oocytes matured in vitro. Nature, **221**, 632–635.

Eppig, J.L. (1993) Regulation of mammalian oocyte maturation. In Adashi, E.Y., Leung, P.C.K. (eds), The Ovary. Raven Press, New York.

Eyestone, W. H., and First, N. L. (1989) Co-culture of early cattle's embryos to the blastocyst stage with oviductal tissue or in conditioned medium. J. Reprod. Fertil., **85**, 715–720.

Franks, S., Mason, H.D., and Polson, D.W. (1988) Mechanism and management of ovulatory failure in women with polycystic ovary syndrome. Hum. Reprod., **3**, 531–534.

Fukuda, Y., Ichikawa, M., Naito, K. et al. (1990) Birth of normal calves resulting from bovine oocytes matured, fertilized, and cultured with cumulus cells in vitro up to the blastocyst stage. Biol. Reprod., **42**, 114–119.

Goto, K., Kajihara, Y., Kosaka, S. et al. (1988) Pregnancies after co-culture of cumulus cells with bovine embryos derived from in-vitro fertilization of in vitro matured follicular oocytes. J. Reprod. Fertil., **83**, 753–758.

Govan, A.D.T. (1970) Ovarian follicular activity in late pregnancy. J. Endocrinol., **48**, 235–241.

Kim, C.I., Ellington J.E., and Foote, R.H. (1990) Maturation, fertilization and development of bovine oocytes in-vitro using TCM199 and a simple defined medium with co-culture. Theriogenology, **33**, 433–440.

Ménézo, Y.J. R., Guerin, J. F. and Czyba, J.C. (1990) Improvement of human early embryo development in-vitro by co-culture on monolayers of Vero cells. Biol. Reprod., **42**, 301–307.

Papaxanthos-Roche, A., Taupin, J.L., Mayer, G. et al. (1994) Human interleukin for DA cells or leukemia inhibitory factor is released by Vero cells in human embryo coculture. Fertil. Steril., **62**, 648–650.

Pfeifer, T.L. and Chegini, N. (1994) Immunocytochemical localization of insulin-like growth factor (IGF-1), IGF-1 receptor and IGF-binding proteins 1-4 in human Fallopian tube at various reproductive stages. Biol Reprod., **50**, 281–289.

Pincus, G. and Enzmann, E.V. (1935) The comparative behavior of mammalian eggs in vivo and in vitro. I. The activation of ovarian eggs. J. Exp. Med., **62**, 665–675.

Pincus, G. and Saunders, B. (1939) The comparative behavior of mammalian eggs in vivo and in vitro. VI. The maturation of human ova. Anat. Rec., **75**, 537–544.

Plachot, M. (1996) Co-culture of embryos and feeder cells. Hum. Reprod., **11** (Suppl. 1), 35–42.

Smotrich, D. B., Stillman, R.J., Widra, E.A. et al. (1996) Immunocytochemical localization of growth factors and their receptors in human pre-embryos and Fallopian tubes. Hum. Reprod., **11**, 184–190.

Starup, J. and Visfeldt, J. (1974) Ovarian morphology in early and late human pregnancy. Acta Obstet. Gynec. Scand., **53**, 211–218.

Tarlatzis, B.C., Grimbizis, G., and Kliniki, G. (1997) The significance of high follicular-phase luteinizing hormone levels in the treatment of women with polycystic ovarian syndrome by in-vitro fertilization. J. Assist. Reprod. Genet., **14**, 1–4.

Trounson, A., Wood, C. and Kausche, A. (1994) In-vitro maturation and fertilization and developmental competence of oocytes recovered from untreated polycystic ovarian patients. Fertil. Steril., **62**, 353–362.

Westergaard, L.G. (1992) Follicular atresia in the human ovary. In Sjöberg, N.-O., Hamberger, L, Janson, P.O. et al. (eds), Local Regulation of Ovarian Function. The Parthenon Publishing Group, Lancs., UK, pp. 55–64.

Westergaard, L.G., McNatty, K.P. and Christensen, I.J. (1985) Steroid concentrations in fluid from human antral follicles during pregnancy. J. Endocrinol., **107**, 133–136.

Westergaard, L.G., Anderson, Y., and Byskov, A.G. (1990) Epidermal growth factor in small antral ovarian follicles of pregnant women. J. Endocrinol., **127**, 363–367.

Wiemer, K.E., Watson, A.J., Polanski, V. et al. (1991) Effects of maturation and co-culture treatments on the developmental capacity of early bovine embryos. Mol. Reprod. Dev., **30**, 330–338.

Wiemer, K.E., Hoffman, D.I., Maxon, W.S. et al. (1993) Embryonic morphology and rate of implantation of human embryos following co-culture on bovine oviductal epithelial cells. Hum. Reprod., **8**, 97–101.