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Maturation *in vitro* of human oocytes from unstimulated cycles: selection of the optimal day for ovum retrieval based on follicular size

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The potential use of immature oocytes for in-vitro fertilization (IVF) requires the conditions for successful maturation to be defined. This study focused on the day of oocyte retrieval. The selection of a dominant follicle may induce endocrine changes in the remaining cohort that may be detrimental to their subsequent fertilization and embryonic development. Natural cycles in volunteer donors were followed by measurement of serum oestradiol and by vaginal ultrasound, starting on day 3 of the cycle. Cycles were randomly allocated to one of two groups: group 1 ($n = 10$), in which follicles were aspirated before the leading follicle was 10 mm in diameter; and group 2 ($n = 9$), in which follicles were aspirated when a dominant follicle was clearly visible with diameter >10 mm. Oocytes were cultured *in vitro* to metaphase II (MII) stage, donated, and inseminated by intracytoplasmic sperm injection (ICSI) with husband's spermatozoa. Those that became fertilized within 24 h were further co-cultured in autologous endometrial epithelial cells up to the blastocyst stage, and cryopreserved. There was a significantly ($P < 0.05$) increased rate of oocyte retrieval in group 1 (70.8% of aspirated follicles) compared with group 2 (50.5%). Maturation to MII and fertilization were similar between the groups. However, development to blastocyst stage was significantly ($P < 0.05$) higher in group 1 embryos (56.5%) compared with group 2 (35.7%). There was a positive correlation ($r^2 = 0.1978$) between the appearance of the cumulus cells and the ability to develop to blastocyst stage when both parameters were analysed in group 1, whereas no such correlation was found in group 2. In conclusion, our data suggest the importance of retrieving immature oocytes before follicular selection, and define the conditions for the first stage in the use of immature oocytes. Further stages must be defined before this technique can be used clinically.

Key words: blastocyst/embryo co-culture/immature oocyte/intracytoplasmic sperm injection/oocyte maturation *in vitro*

Introduction

Maturation *in vitro* of oocytes has been investigated in different mammalian species, and has not only resulted in embryonic development (Schroder and Epigg, 1984) but has also been applied commercially, such as the improvement of fertility in cattle (Looney *et al*., 1994; Trounson *et al*., 1994a).

The technique of maturation *in vitro* of human oocytes is an attractive option for the treatment of infertility. It would enable a decrease in the use of ovarian stimulation drugs in normo- and anovulatory patients, especially those with polycystic ovaries (PCO), who are at risk of developing ovarian hyperstimulation syndrome. Another possible application is related to cryopreservation. It is known that the freezing of oocytes in the metaphase II (MII) stage damages the genetic material. Oocytes in germinal vesicle (GV) stage have their DNA protected by the nuclear membrane, and animal studies have shown the protective effect of the surrounding cumulus cells (Pellicer *et al*., 1988). Thus, freezing immature oocytes may be an excellent alternative for patients who wish to postpone their reproductive function, for women undergoing treatment with chemotherapy or radiation for cancer, or for the creation of banks for ovum donation.

Although some work has already been carried out in humans, there has been no systematic analysis of the subject. Acceptable rates of maturation, fertilization, embryonic development and term pregnancies have been reported employing immature oocytes from stimulated cycles (Veeck *et al*., 1983; Nagy *et al*., 1996; Jaroudi *et al*., 1997). Similarly, pregnancies and newborn infants have been obtained after maturation *in vitro* of oocytes recovered following ovariectomies (Cha *et al*., 1991; Cha and Chiang, 1998), from PCO patients (Trounson *et al*., 1994b; Barnes *et al*., 1995), or from women undergoing intracytoplasmic sperm injection (ICSI) in natural cycles (Russell *et al*., 1996). These results show that, if oocyte retrieval procedures are optimized and adequate techniques for maturation *in vitro* and fertilization are developed, it may be possible to consider the use of immature oocytes in a clinical setting.

Healthy follicles measuring 2–5 mm—referred to as selectable follicles—are observed at all stages of the cycle. Those present during the late luteal phase constitute the population from which the follicle destined for ovulation during the subsequent cycle will be selected (McNatty *et al*., 1983; Gougeon, 1996). The newly selected follicle belongs to the class of follicles measuring 5–8 mm, and its size increases greatly during the follicular phase by cellular multiplication and accumulation of fluid in the antrum. Thus, from a clinical standpoint it has been considered that the dominant follicle can be easily recognized by ultrasound when its diameter has reached 10 mm (Fauser and van Heusden, 1997).

The intrafollicular steroid milieu of the follicle changes dramatically when selection has been completed and dominance begins. Healthy and atretic follicles have a similar milieu characterized by a low oestrogen/androgen ratio, although the concentration of androgens present in healthy follicles is low because there is only limited expression of enzymes such as $P450_{scc}$ and $P450_{17\alpha}$ hydroxylase (Sasano *et al*., 1989; Tamura *et al*., 1992). When selection takes place, there is a shift in oestrogen/androgen ratio in the dominant follicle, while the other follicles remain androgenized and become atretic. Thus, although the androgen influence does not disappear from the non-dominant follicles, the period of exposure to androgens and the possible (unknown) increase in androgen concentrations during the follicular phase may cause a degree of oocyte damage.

In order to define the optimal conditions for the use of human immature oocytes in clinical practice, we have divided the technique into several distinct steps: oocyte retrieval, storage, maturation *in vitro*, fertilization and development, and implantation. This study explores the first step. Our hypothesis was that the time of ovum retrieval, whether before or after obvious follicular selection, may make a difference to the quality and developmental potential of human oocytes following in-vitro maturation. To test our hypothesis, a prospective study was designed which employed natural cycles from volunteer donors.

Materials and methods

Recovery and culture of immature oocytes

Sixteen women (19 cycles) from our oocyte donation programme, whose ages ranged from 23 to 32 years (mean ± SEM age 24.5 ± 3.6 years) were included in this study. Each natural cycle was monitored every 48 h by transvaginal ultrasound and measurement of serum oestradiol concentration, starting on cycle day 3. The cycles were randomly allocated to one of two groups: group 1 ($n = 10$), in which follicles were aspirated before the leading follicle was 10 mm in diameter; and group 2 ($n = 9$) in which at least one follicle was >10 mm in diameter when follicles were aspirated.

Follicle aspiration was performed by ultrasound-guided transvaginal puncture, employing regular IVF needles (Swemed Laboratories, Billdal, Sweden; 1.6×350 mm, 16 g) and 80 mmHg aspiration pressure. The system was previously washed with flushing heparinized medium (Medicult, Copenhagen, Denmark), and the follicular fluid collected in 2 ml of the same medium in sterile plastic tubes.

Oocytes were examined under the dissecting microscope and classified according to cumulus appearance as type 1, with few surrounding granulosa cells; type 2, with a moderate number of granulosa cells; and type 3, with several layers of granulosa cells surrounding each oocyte.

Oocytes were cultured in M-199 medium (Sigma, St Louis, MO, USA), supplemented with 0.4% HSA and 0.33 mM pyruvate, at 37°C in a 5% CO_2 atmosphere. Hormones were added as follows: 0.075 IU/ml recombinant follicle stimulating hormone (FSH) (Gonal-F; Serono Laboratories, Madrid, Spain); 2 ng/ml recombinant endothelial growth factor (EGF) (Sigma); 0.5 IU/ml human chorionic gonadotrophin (HCG) (Profasi; Serono); and 1 μg/ml oestradiol (Sigma). After 24–36 h, cumulus cells were removed by pipetting, and oocyte nuclear maturation was evaluated from the presence of the first polar body.

Table I. Cycle characteristics in the two groups of patients defined by follicular diameter

	Group 1	Group 2
No. of cycles	10	9
Age (years)	24.1 ± 3.6	25.0 ± 3.5
Follicular diameter (mm) (range)	7.6 ± 1.2 (5–9)	11.4 ± 0.5 (9–14)*
Day of retrieval (Day 0)	7.0 ± 1.6	9.2 ± 0.6
No. of oocytes (retrieval rate, %)	68 (70.8)	44 (50.5)*
Oestradiol (pg/ml), day –2	48.1 ± 4.7	69.3 ± 3.0*
Oestradiol (pg/ml), day 0	48.9 ± 3.4	89.4 ± 3.9*

*$P < 0.05$.
Values are means ± SEM.

Fertilization and embryo development

Oocytes reaching MII stage were donated to women on the waiting list of our oocyte donation programme. Each was microinjected by ICSI with husband's spermatozoa that were normozoospermic in all cases. The ICSI procedure has been described in detail elsewhere (Gil-Salom *et al*., 1995). Fertilization was evaluated after 18 h of culture under standard conditions (37°C, 5% CO_2) from the presence of two pronuclei and two polar bodies. Embryos at the 2–4-cell stage were co-cultured with human autologous endometrial epithelial cells as described previously (Simon *et al*., 1997, 1999). Embryo development was evaluated as far as the blastocyst stage, and those reaching this stage of development were cryopreserved.

Oestradiol concentrations

Serum oestradiol concentrations were measured using commercially available radio-immunoassay kits (bioMérieux, Charbonniéres les Bains, France). The inter- and intra-assay variability for oestradiol at a concentration of <40 pg/ml were 2.8% and 4.3% respectively.

Statistical analysis

Results are expressed as mean ± SEM. Comparative analysis of quantitative variables such as age, puncture day and serum oestradiol concentrations was made by ANOVA. Categorical and or dichotomised variables, i.e. follicular diameter, maturation, fertilization and development to blastocyst, were analysed by χ^2 test and Fisher's exact test. To compare the number of oocytes in each group, the binomial test was performed. In the comparative analysis of the ordinal variable 'type of cumulus', the Mann–Whitney *U*-test was used. Three dichotic variables (maturation, fertilization and development to blastocyst) were grouped in one single ordinal variable named 'oocyte development degree' with the purpose of adding more power to the statistical calculations. Spearman's correlation coefficient (r) was employed to study the relationship between the two ordinal variables 'type of cumulus' and 'oocyte development degree'. Significance was defined as $P < 0.05$.

Results

Follicular aspiration in the 19 unstimulated cycles occurred on day 7.7 ± 1.7. A total of 112 oocytes was retrieved, giving a recovery rate of 61.1% from the total number of antral follicles punctured. Two oocytes were atretic, 51 (46.4%) reached MII stage, 37 (72.6%) became fertilized after ICSI, and 18 (48.6% of zygotes) reached the blastocyst stage.

Table I shows the results obtained in the two groups defined according to follicular diameter. Follicular size was significantly higher in group 2 ($P < 0.05$), in which ovum

Table II. Oocyte maturation, fertilization and embryo development *in vitro*

	Group 1	Group 2
No. of cycles	10	9
Atretic oocytes	1	1
Type of cumulus		
Few granulosa cells	23 (34.3)	16 (36.4)
Moderate granulosa cells	18 (26.9)	17 (38.6)
Multilayered	26 (38.8)	10 (25.0)
Metaphase II	35 (52.2)	16 (37.2)
Fertilization	23 (65.7)	14 (87.5)
Blastocysts	13 (56.5)	5 (35.7)*

*$P < 0.05$.
Values in parentheses are percentages.

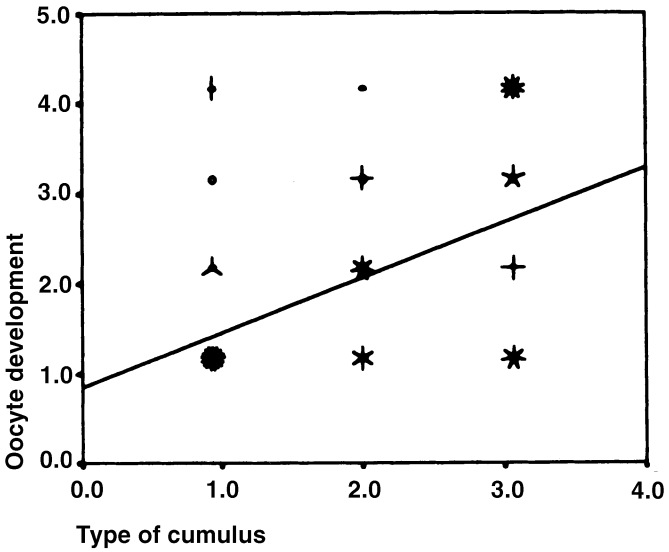

Figure 1. Scatter diagram comparing embryo development and type of cumulus for group 1. At each point the number of oocytes is represented by the central circle plus the number of 'tails' radiating outwards. The regression line shows a positive correlation between variables ($r^2 = 0.1978$). On the vertical axis, scale values are: 1 = oocytes that did not mature *in vitro*; 2 = oocytes that matured *in vitro*; 3 = oocytes that became fertilized; 4 = oocytes that reached blastocyst stage. On the horizontal axis, the type of cumulus is represented as: 1 = few granulosa cells; 2 = several layers of granulosa cells; 3 = abundant granulosa cells.

aspiration occurred two days later than in group 1. The retrieval rate was 70.8% in group 1 and 50.5% in group 2 ($P < 0.05$). Serum oestradiol concentrations were significantly ($P < 0.05$) higher in group 2 than in group 1, as an indication of a more advanced stage of folliculogenesis.

The outcome of oocyte maturation and fertilization is shown in Table II. Oocyte quality as ascertained by the number of cumulus cells surrounding the oocyte, the percentage of atretic oocytes, and the rate of maturation to MII, was similar in the two groups. Similarly, the rate of fertilization by ICSI did not differ. There was however, a significantly ($P < 0.05$) higher proportion of zygotes reaching the blastocyst stage in co-culture in group 1 compared with group 2.

This analysis is shown in scatter diagrams (Figures 1 and 2) which correlate the degree of embryo development and the type of cumulus surrounding the oocyte in the two groups of patients. Figure 1 indicates a positive correlation between the

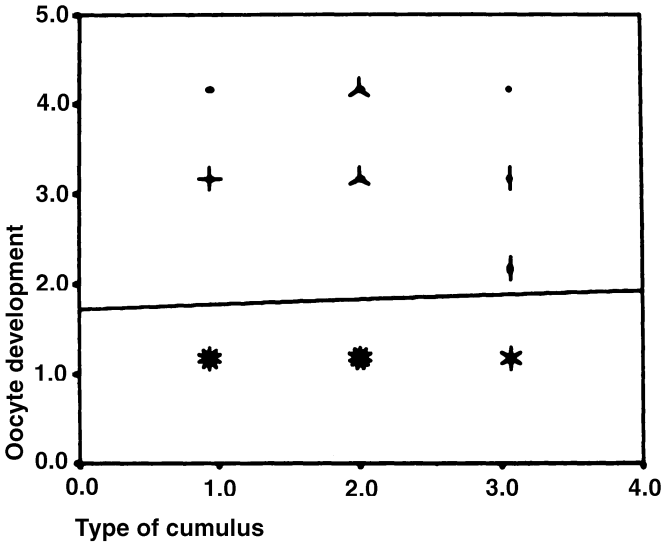

Figure 2. Scatter diagram comparing embryo development and type of cumulus for group 2. At each point the number of oocytes is represented by the central circle plus the number of 'tails' radiating outwards. The regression line indicates no correlation between variables ($r^2 = 0.0011$). Details of the vertical and horizontal axes are as Figure 1.

presence of multiple layers of granulosa cells in the cumulus and the ability of the embryos to develop. This correlation was not found in the oocytes obtained in group 2 (Figure 2).

Discussion

This study shows that the day of retrieval of human oocytes is a critical parameter for successful outcome in terms of maturation *in vitro* and subsequent development up to the blastocyst stage. Our data show that once selection of the leading follicle has occurred, the number of retrievable human oocytes decreases and their ability to undergo fertilization and development is impaired. We believe that these findings reflect the endocrine milieu found within the cohort of follicles during follicular dynamics. Follicular selection induces a predominantly oestrogenic milieu in the dominant follicles, whereas those destined to become atretic maintain an androgenic environment that, in turn, may irreversibly damage the oocytes. As a result, we found a lower rate of development into blastocysts after selection, and no correlation between cumulus quality and embryo development, suggesting that the cells surrounding the oocyte may also be affected by the process of cellular death.

Although aromatase activity is initially low in follicles from the cohort before selection, in-vitro production of oestradiol can be rapidly stimulated by adding FSH to the culture medium (Hillier *et al.*, 1980; Mason *et al.*, 1994). Oestradiol may in turn act upon the oocyte, as detection of oestradiol receptor mRNA in the human oocyte has been confirmed by Southern blot analysis (Wu *et al.*, 1993). In addition, it has been shown that the addition of oestradiol to oocyte maturation medium can directly influence the quality of the maturing oocyte (Tesarik and Mendoza, 1995). Other hormones and growth factors may also play a role in the process of maturation

Table III. Results of previous reports using immature human oocytes from unstimulated cycles

Reference	No. of oocytes	% MII	Fertilization rate (%)	Cleavage rate (%)	Blastocyst rate (%)	Embryo transfer	No pregnancy
Carson (1997)	109	59.6	63.5[a]	75.6	–	Yes	1
Knezevich *et al.* (1996)	100	61.0	73.8[a]	–	–	No	–
Barnes *et al.* (1996)	234	67.0	32.0[b]	19.0	–	No	–
Russell *et al.* (1995)	161	50.9	74.3[a]	59.0	–	Yes	1
Barnes *et al.* (1995)	52	57.6	43.3[c]	84.6	–	Yes	1
Carson *et al.* (1995)	36	63.8	47.8[a]	–	–	Yes	1
Trounson *et al.* (1994b)	403	50.6	40.0[b]	56.0	–	Yes	1
Present study	112	46.4	77.0[a]	81.6	48.6	No	–
Total	1207	57.1	56.5	62.6	48.6	–	5

[a]ICSI; [b]IVF; [c]IVF/ICSI.

in vitro. We have shown in rodents (Pellicer *et al.*, 1989) and humans (Gómez *et al.*, 1993) that EGF induces oocyte maturation *in vitro*, and this growth factor was also included in our culture system.

Thus, we believe that the hormones added to the culture medium in group 1 oocytes exerted a positive effect on their meiotic and cytoplasmic competence. Evidence suggests that once the dominant follicle is selected, granulosa cells become less receptive to FSH (Fauser and van Heusden, 1997) and perhaps other hormones, and this may reflect the situation in group 2 oocytes.

Maturation rates did not differ between groups. This observation is not surprising because it has long been recognized that oocytes removed from their follicles can undergo nuclear maturation with extrusion of the first polar body (Pincus and Enzmann, 1935; Pincus and Saunders, 1939; Edwards, 1965). This is also true for atretic follicles, both *in vitro* and *in vivo* (Gougeoun and Testart, 1986). Similarly, fertilization rates by ICSI were similar. We believe that this observation confirms the power of ICSI, and suggests that there may be various factors related to the acquisition of fertilization and cleavage within the cytoplasm of the oocytes.

The high rate of blastocyst formation observed in group 1 (56.5%) is an interesting point of discussion. Cytoplasmic maturation is closely linked to all those processes that prepare the oocytes for fertilization, activation and embryo development. Evidence from goat, murine and bovine studies suggests that the capacity to reach blastocyst stage is higher for oocytes obtained from larger antral follicles than for oocytes from smaller follicles (Pavlok *et al*., 1992; Eppig and Wigglesworth, 1994; Crozet *et al*., 1995). Hence, nuclear and cytoplasmic maturation may occur in a coordinated fashion in larger follicles. In group 1 of the present study, all selected follicles were healthy and belonged to the class of larger non-dominant follicles where differentiation of GV stage oocytes could be related to acquisition of competence to undergo pre-implantational development.

We employed a simplified method for ovum retrieval using routine IVF tools, and obtained an acceptable retrieval rate of 5.9 oocytes per patient (61.1%). However, this was lower than the rate obtained using special needles for ovum retrieval (Trounson *et al.*, 1994b; Russell *et al.*, 1996).

Table III summarizes the results of several reports concerning the outcome of 1207 in-vitro-matured oocytes obtained from unstimulated cycles in terms of maturation rate to MII stage, fertilization and cleavage rates, and pregnancies. Metaphase II stage oocytes obtained in different studies are similar, but there is more variability in fertilization and cleavage rates. This might be the consequence of differences in the developmental capability of the oocytes, which have not been obtained at the same stage of the cycle. Also, different culture conditions may influence the acquisition of cytoplasmic factors affecting oocyte competence to undergo fertilization and successful embryogenesis. Our fertilization and cleavage rates are higher than other reported figures, and the high rate of blastocyst formation (48.6%) suggests that collecting oocytes at the right time, and placing them in an optimal environment, might improve embryo quality.

In conclusion, our results suggest that follicular diameter at the time of aspiration plays an important role in the development of the immature oocytes recovered in a natural cycle, and may be a relevant parameter in the development of a technology which permits successful oocyte maturation *in vitro*. Follicular growth must be monitored carefully to ensure that ovum retrieval takes place before the follicular diameter exceeds 10 mm (day 7.0 $\pm$ 1.2 of the cycle). Thus, the initial optimal conditions have been established. Further work is needed to define the best conditions for cryopreservation, maturation, fertilization, development *in vitro*, and implantation after replacement, before the clinical use of immature oocytes.

References

Barnes, F., Crombie, A., Gardner, D. *et al.* (1995) Blastocyst development and birth after *in vitro* maturation of human primary oocytes, intracytoplasmic sperm injection and assisted hatching. *Hum. Reprod.*, **10**, 3242–3247.

Barnes, F., Kausche, A., Tiglias, J. *et al.* (1996) Production of embryos from *in vitro* matured human oocytes. *Fertil. Steril*., **65**, 1151–1156.

Carson, R. (1997) Maturation of human oocytes *in vitro*. *J. Assist. Reprod. Genet*., **14** (abstract book), p. 431.

Carson, R., Stangel, J., Crumm, K. *et al*. (1995) Maturation of oocytes *in vitro*. *Fertil. Steril*., Annual Meeting Program Supplement, O-075, S37.

Cha, K. and Chiang, R.C. (1998) Maturation *in vitro* of immature human oocytes for clinical use. *Hum. Reprod. Update*, **4**, 103–120.

Cha, K., Koo, J., Ko, 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.

Crozet, N., Ahmed-Ali, M. and Dubos, M.P. (1995) Developmental competence of goat oocytes from follicles of different size categories following maturation, fertilisation and culture *in vivo*. *J. Reprod. Fertil*., **103**, 293–298.

Edwards, R. (1965) Maturation *in vitro* of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. *Nature*, **102**, 493–497.

Eppig, J.J. and Wigglesworth, K. (1994) Atypical maturation in oocytes in strain I/LnJ mice. *Hum. Reprod*., **9**, 1136–1142.

Fauser, B. and van Heusden, A. (1997) Manipulation of human ovarian function: physiological concepts and clinical consequences. *Endocr. Rev*., **1**, 71–103.

Gil-Salom, M., Minguez, Y., Rubio, C. *et al*. (1995) Intracytoplasmic testicular sperm injection: an effective treatment for otherwise intractable obstructive azoospermia. *J. Urol.*, **154**, 2074–2077.

Gómez, E., Tarín, J.J. and Pellicer, A. (1993) Oocyte maturation in humans: the role of gonadotropins and growth factors. *Fertil. Steril*., **60**, 40–46.

Gougeoun, A. (1996) Regulation of ovarian follicular development in primates: facts and hypotheses. *Endocr. Rev*., **2**, 121–155.

Gougeoun, A. and Testart, J. (1986) Germinal vesicle breakdown in oocytes of human atretic follicles during the menstrual cycle. *J. Reprod. Fertil.*, **78**, 389–401.

Hillier, S.G., van der Boagaard, A., Reichert, L.E., Jr and van Hall, E.V. (1980) Intraovarian sex steroid hormone interactions and the regulation of follicular maturation: aromatization of androgens by human granulosa cells *in vitro*. *J. Clin. Endocrinol. Metab.*, **50**, 640–647.

Jaroudi, K., Hollanders, J.M.G., Sieck, U. *et al*. (1997) Pregnancy after transfer of embryos which were generated from *in vitro* matured oocytes. *Hum. Reprod*., **12**, 857–859.

Knezevich, K.M., Russell, B., Fabian, K.F. *et al*. (1996) Analysis of *in vitro* oocyte maturation and fertilization results. *Fertil. Steril*., Annual Meeting Program Supplement, O-019, S10.

Looney, C., Lindsay, B., Gonseth, C. and Johnson, D. (1994) Commercial aspects of oocyte retrieval and *in vitro* fertilization for embryo production problem in cow. *Theriogenology*, **41**, 67–72.

Mason, H.D., Wills, D., Beard, R. *et al*. (1994) Estradiol production by granulosa cells of normal and polycystic ovaries: relationship to menstrual cycle history and concentration of gonadotropins and sex steroids in follicular fluids. *J. Clin. Endocrinol. Metab.*, **79**, 1355–1360.

McNatty, K.P., Hillier, S.G., van den Boogaard, A.M.J. *et al*. (1983) Follicular development during the luteal phase of the human menstrual cycle. *J. Clin. Endocrinol. Metab*., **56**, 1022–1031.

Nagy, Z., Cecile, J., Liu, J. *et al*. (1996) Pregnancy and birth after intracytoplasmic sperm injection of *in vitro* matured germinal vesicle stage oocytes: case report. *Fertil. Steril.*, **65**, 1047–1050.

Pavlok, A., Lucas-Hahn, A. and Niemann, H. (1992) Fertilisation and developmental competence of bovine oocytes derived from different categories of antral follicles. *Mol. Reprod. Dev*., **31**, 63–67.

Pellicer, A., Lightman, A., Parmer, T.G. *et al*. (1988) Morphological and functional studies of immature rat oocyte–cumulus complexes after cryopreservation. *Fertil. Steril.*, **50**, 805–810.

Pellicer, A., Parmer, T.G., Stoane, J.M. and Behrman, H.R. (1989) Desensitization to follicle-stimulating hormone in cumulus cells is coincident with hormone induction of oocyte maturation in the rat follicle. *Mol. Cell. Endocrinol*., **64**, 179–188.

Pincus, G. and Enzmann, E. (1935) The comparative behaviour of mammalian eggs *in vivo* and *in vitro*. I. The activation of ovarian eggs. *J. Exp. Med*., **62**, 655–675.

Pincus, G. and Saunders, B. (1939) The comparative behaviour of mammalian eggs *in vivo* and *in vitro*. IV. The maturation of human ovarian ova. *Anat. Rec*., **75**, 537–545.

Russell, J.B., Knezevich, K.M., Fabian, K.F. *et al*. (1995) Unstimulated immature oocyte retrieval: early vs mid follicular endometrial priming. *Fertil. Steril*., Annual Meeting Program Supplement, O-1, A1.

Russell, J.B., Knezevich, K.M., Fabian, K.F. *et al*. (1996) Unstimulated immature oocyte retrieval: early vs midfollicular endometrial priming. *Hum. Reprod.*, **11** (Abstract Book 1), 2.

Sasano, H., Okamoto, M., Mason, J.I. *et al*. (1989) Immunolocalization of aromatase, 17α-hydroxylase and side chain cleavage cytochrome $P\text{-}_{450}$ in the human ovary. *J. Reprod. Fertil.*, **85**, 163–169.

Shroder, A. and Eppig, J. (1984) The developmental capacity of mouse oocytes that matured spontaneously *in vitro* is normal. *Dev. Biol.*, **102**, 493–497.

Simon, C., Gomeno, M.J., Mercader, A. *et al*. (1997) Embryonic regulation of integrins β_3, α_3, and α_1, in human endometrial epithelial cells *in vitro*. *J. Clin. Endocrinol. Metab.*, **82**, 2607–2616.

Simon, C., Mercader, A., García-Velasco, J. *et al*. (1999) Coculture of human embryos with autologous endometrial epithelial cells in patients with implantation failure. *J. Clin. Endocrinol. Metab.* (in press).

Tamura, T., Kitawaki, J., Yamamoto, T. *et al*. (1992) Immunohistochemical localization of 17α-hydroxylase/C17-20 lyase and aromatase cytochrome $P\text{-}_{450}$ in the human ovary during the menstrual cycle. *J. Endocrinol*., **135**, 589–595.

Tesarik, J. and Mendoza, C. (1995) Nongenomic effects of 17β-estradiol on maturing human oocytes: relationship to oocyte developmental potential. *J. Clin. Endocrinol. Metab*., **80**, 1438–1443.

Trounson, A., Pushet, D., MacLellan, L. *et al*. (1994a) Current status of IVM/IVF and embryo culture in humans and farm animals. *Theriogenology*, **41**, 57–66.

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

Veeck, L., Edward, J., Witmyer, J. *et al*. (1983) Maturation and fertilization of morphologically immature oocytes in a program of *in vitro* fertilization. *Fertil. Steril.*, **39**, 594–602.

Wu, Tch J., Wang, L. and Wan, Y.J.Y. (1993) Detection of estrogen receptor messenger ribonucleic acid in human oocytes and cumulus–oocyte complexes using reverse transcriptase–polymerase chain reaction. *Fertil. Steril.*, **59**, 54–59.

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