Possible factors affecting the development of oocytes in in-vitro maturation

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To date, pregnancy rates from oocytes matured in-vitro (IVM) have been much lower than those with in-vivo stimulated maturation. In order to improve the developmental potential of IVM oocytes, we studied the effect of three possible factors on pregnancy rates: (i) priming \textit{in vivo} with FSH before aspiration; (ii) the time interval of maturation \textit{in vitro}, and (iii) timing the aspiration by monitoring the serum concentrations of oestradiol and inhibin A. In all experiments, oocyte retrieval was performed transvaginally and oocytes were matured individually in culture medium (TCM 199) under oil. Intracytoplasmic sperm injection (ICSI) was carried out on all metaphase II oocytes. Suitable embryos (maximum of two) were replaced after culturing for 2–3 days in IVF medium. Endometrial priming consisted of 2 mg 17\textbeta-oestradiol, taken orally three times a day from the day of oocyte retrieval, and intravaginal progesterone suppositories initiated 2 days later. In the first experiment, 20 women were randomly allocated to two groups: group I (n = 10 cycles) received no stimulation, while group II (n = 10 cycles) received recombinant FSH 150 IU/day for 3 days, initiated on day 3. FSH priming did not affect the rates of maturation, fertilization or cleavage, and no effect was seen on embryo development. The second experiment included 48 patients undergoing 55 unstimulated cycles. The effects of IVM periods of 28 and 36 h were compared. Shortening the IVM period did not compromise subsequent embryo development. The third study analysed the results of maturation of oocytes obtained in 87 cycles in 75 unstimulated normal women, after a leading follicle of 10 mm in diameter and an endometrial thickness of at least 5 mm were observed. A pregnancy rate of 12.6% (11/87) per aspiration and 17.4% (11/63) per transfer was obtained. Serum concentrations of oestradiol and inhibin A were evaluated retrospectively. Significantly more pregnancies were obtained in cycles with a detected increase in the concentration of oestradiol from day 3 to the day of aspiration (19\% per aspiration) compared with cycles without such an increase (0\% per aspiration). A higher pregnancy rate was observed after an increase in inhibin A concentration (24 versus 0\%). In conclusion, monitoring the timing of aspiration may potentially improve the developmental potential of immature oocytes. FSH priming did not affect either cleavage rate or embryo development. Shortening the maturation period from 36 to 28 h did not compromise subsequent embryonic development.

Key words: FSH priming/human oocytes/in-vitro maturation/maturation time/timing of aspiration

Introduction

In future, immature oocyte retrieval combined with in-vitro maturation (IVM) could possibly replace standard stimulated IVF. Clinically, IVM would simplify the process of obtaining oocytes for repro-
duction by avoiding or minimizing the need to carry out ovarian stimulation (Cha and Chiang, 1998; Russell, 1998; Trounson et al., 1998; Mikkelsen et al., 1999; Chian et al., 2000; Suikkari et al., 2000), and therefore the technique may reduce the costs and avert the side-effects of gonadotrophins.

Maturation of human oocytes has two aspects: (i) nuclear maturation leading to extrusion of the first polar body; and (ii) cytoplasmic maturation which allows the protein synthesis required for normal fertilization and embryo development. When comparing oocytes matured in vivo with those matured in vitro, no major differences can be seen in the level of nuclear maturation, or in the fertilization and cleavage rates, but pregnancy rates from oocytes matured in vitro are much lower than those of in-vivo stimulated cycles, indicating that embryo viability is compromised (Cha et al., 1991; Trounson et al., 1994; Barnes et al., 1996; Russell et al., 1997). There are several possible explanations for the poor developmental capacity of these oocytes, including suboptimal culture conditions during IVM or the possibility that the oocytes themselves were defective, due to inadequate cytoplasmic maturation (Moor et al., 1998).

We investigated normal, regularly menstruating, women who had been referred for IVF/ICSI because of male factor infertility and/or tubal disease. The women were aged 18–37 years and had normal ovulatory cycles with a mean duration of 26–35 days and a body mass index (BMI) of 18–29 kg/m². Patients were excluded if infertility was caused by endocrine abnormalities, e.g. hyperprolactinaemia, and patients with an expected low ovarian reserve evaluated on day 3 by an antral follicle count of three or less at 2–5 mm and/or an FSH concentration of >15 IU, and/or an inhibin B concentration of <45 pg/ml. Patients who had undergone more than three failed IVF attempts were also excluded, and patients with possible poor quality of the oocytes, i.e. patients with a low <20% cleavage rate at conventional IVF and women with polycystic ovary syndrome (PCOS). PCOS was defined by ultrasound examination showing >10 follicles in one plane, and hormone analysis showing an elevated LH/FSH ratio or elevated androgens.

Follicle development in the ovaries was monitored by transvaginal ultrasound examination beginning on cycle day 3 and oocytes were aspirated after the leading follicle had reached 10 mm in diameter. The same observer measured the follicular diameters during transvaginal ultrasound scanning using a 7.5 MHz transvaginal transducer (B-K Medical, Gentofte, Denmark). The follicular diameter was calculated as the mean of the longest follicular axis and an axis perpendicular to it.

Endometrial priming consisting of 17β-oestradiol started on the day of oocyte retrieval and the women received 2 mg orally three times per day. Two days after aspiration, treatment with intravaginal progesterone suppositories (3×100 g Progestan; Organon, Oss, The Netherlands) was initiated and continued until the pregnancy test. Oestrogen and progesterone were continued if the pregnancy test was positive until 50 days gestation. The local ethical committee approved the studies and all participating couples gave written consent.

**Materials and methods**

**Patients**

The experiments included healthy women referred for IVF/ICSI because of male factor infertility and/or tubal disease. The women were aged 18–37 years and had normal ovulatory cycles with a mean duration of 26–35 days and a body mass index (BMI) of 18–29 kg/m². Patients were excluded if infertility was caused by endocrine abnormalities.
Oocytes with signs of atresia (dark or shrunken, irregular cytoplasm) or mechanical damage were discarded. Oocytes which appeared healthy, with multi-layered or sparse cumulus, were matured in TCM 199 (Sigma, Roedovre, Denmark). The culture medium was supplemented with 0.3 mmol/l sodium pyruvate, 1500 IU/ml penicillin G, 50 mg/ml streptomycin sulphate, and 1 µg/ml oestradiol (all from Sigma), 0.075 IU/ml recombinant FSH (rFSH, Gonal-F; Serono, Geneva, Switzerland), 0.5 IU/ml human chorionic gonadotrophin (HCG, Profasi; Serono) and serum from the patient (10%). The oocytes were cultured singly in 25 µl drops of IVM medium under paraffin oil at 37°C in 5% CO₂ and humidified air.

The presence of the first polar body, which was confirmed microscopically, was the criterion used to classify oocytes as matured to metaphase II. All metaphase II oocytes were inseminated by ICSI and then cultured individually in 10 µl droplets of IVF medium (Medi-Cult) in Falcon petri dishes (Life Technologies) under oil in 5% CO₂ and humidified air at 37°C. Embryos were cultured to day 2.5 or 3 (day 0 = day of insemination), at which time suitable embryos (maximum 2) were transferred into the patient’s uterus.

**Experimental groups**

**Experiment 1: FSH priming**

A total of 20 women were randomly allocated to two groups. Group I (n = 10 cycles) received no stimulation, group II (n = 10 cycles) received rFSH (Gonal-F; Serono) 150 IU/day for 3 days initiated on day 3. All the oocytes were matured in vitro for 36 h before ICSI.

**Experiment 2: maturation period**

A total of 48 women underwent 55 cycles, and were allocated treatment according to their order of entry into the study. In group 1, (21 patients; 29 cycles), the oocytes were inseminated 28 h after the start of aspiration. In group 2 (21 patients; 26 cycles), the oocytes were inseminated 36 h after the start of aspiration.

**Experiment 3: timing of aspiration**

Since the data from the two groups in experiment 2 were very similar, all data from the experiment were pooled, and an additional 32 aspirations were performed (n = 87 cycles). Oocyte retrieval was scheduled when the leading follicle had achieved a diameter of 10 mm and an endometrial thickness of at least 5 mm was observed at ultrasound. The serum concentrations of oestradiol and inhibin A were measured from day 3 to the day of aspiration and evaluated retrospectively.

**Statistical analyses**

Fisher’s exact test or χ² test were used for statistical analyses. Because none of the hormone variables displayed a normal distribution, the non-parametric Mann–Whitney U-test was used to analyse differences between unpaired data and Pratt’s test was used for paired data. P < 0.05 was considered to be statistically significant.

**Results**

**First experiment: priming with FSH**

FSH priming did not show any significant effect on oocyte maturation, fertilization rate, cleavage rate or pregnancy rate (Table I).

**Second experiment: time interval for maturation**

Similar rates of maturation to metaphase II were observed for oocytes that underwent 28 or 36 h of maturation before insemination (73 and 77% respectively). After insemination, 72% of the injected ova were fertilized in group 1 (28 h of maturation) compared with 78% in group 2 (36 h of maturation). The cleavage rates of fertilized ova were 89 and 96% and the pregnancy rates were 14 and 15% in groups 1 and 2 respectively. None of the differences were statistically significant (Table II).

**Third experiment: timing of aspiration**

In total, 532 oocytes were aspirated of which 388 were cumulus enclosed and could be used for IVM. ICSI was carried out on 234 oocytes and fertilization with two pronuclei (2PN) was obtained in 180 (77%). A total of 156 cleaved embryos were obtained (87%) and 125 were used for transfer. In all, 11 singleton gestations with a live fetus were obtained, giving a clinical pregnancy rate per aspiration of 12.6% (11/87), a pregnancy rate per transfer of 17.4% (11/67) and an implantation rate of 8.8% (11/125). Nine healthy children have been
Table I. Numbers of oocytes obtained for in-vitro maturation (IVM), maturation rate, fertilization rate, and cleavage rate in experiment 1 evaluating the effect of FSH priming

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>Oocytes for IVM</th>
<th>MII (%)</th>
<th>2PN (%MII)</th>
<th>Cleavage (%MII)</th>
<th>Pregnancies</th>
</tr>
</thead>
<tbody>
<tr>
<td>No FSH (36 h IVM)</td>
<td>10</td>
<td>37</td>
<td>28 (76)</td>
<td>20 (71)</td>
<td>3</td>
</tr>
<tr>
<td>+ FSH (36 h IVM)</td>
<td>10</td>
<td>40</td>
<td>34 (85)</td>
<td>25 (74)</td>
<td>2</td>
</tr>
</tbody>
</table>

MII = metaphase II; 2PN = fertilization with two pronuclei.

Table II. The maturation rate, fertilization rate, cleavage rate and pregnancy rate after in-vitro maturation (IVM) of oocytes for 28 and 36 h respectively

<table>
<thead>
<tr>
<th>No. of cycles</th>
<th>No. of oocytes cultured</th>
<th>MII (%)</th>
<th>2PN (%MII)</th>
<th>Cleavage (%MII)</th>
<th>Pregnancies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (28 h IVM)*</td>
<td>29</td>
<td>107</td>
<td>78 (73)</td>
<td>56 (72)</td>
<td>4 (14)</td>
</tr>
<tr>
<td>Group 2 (36 h IVM)*</td>
<td>26</td>
<td>84</td>
<td>65 (77)</td>
<td>51 (78)</td>
<td>4 (15)</td>
</tr>
</tbody>
</table>

MII = metaphase II; 2PN = fertilization with two pronuclei.
*There were no statistically significant differences between the groups.

Table III. The maturation, fertilization and cleavage rates of oocytes obtained with or without a >100% increase in the oestradiol concentration between day 3 and the day of oocyte retrieval (n = 85 cycles)

<table>
<thead>
<tr>
<th>No. aspirations</th>
<th>No. oocytes for IVM</th>
<th>MII (%)</th>
<th>2PN (%MII)</th>
<th>Cleavage (%MII)</th>
<th>Pregnancies</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥100% increase</td>
<td>57</td>
<td>258</td>
<td>160 (62)</td>
<td>108 (87)</td>
<td>11 (19)*</td>
</tr>
<tr>
<td>&lt;100% increase</td>
<td>28</td>
<td>115</td>
<td>68 (59)</td>
<td>43 (90)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

IVM = in-vitro maturation; MII = metaphase II; 2PN = fertilization with two pronuclei.
*P < 0.02 (Fisher’s exact test).

born, and two women underwent abortions at weeks 8–9 of gestation.

In 85 cycles, the oestradiol concentration was assessed retrospectively on day 3 and on the day of aspiration. An increase of 100% in the concentration of oestradiol was observed in 57 cycles and an increase of <100% was seen in 28 cycles. Significantly more pregnancies were obtained in cycles with a >100% increase (11/57, 19% per aspiration, 24% per transfer) compared with cycles with <100% increase (0/28, 0 and 0% respectively), see Table III.

The concentration of inhibin A was obtained on day 3 and on the day of aspiration in 83 cycles. In 42 of these cycles, a detected oestradiol increase of at least 100% plus a detected increase in inhibin A concentration of at least 80% were observed on the day of aspiration compared with day 3. In this group, 10 pregnancies were obtained [pregnancy rate 10/42 (24%) per aspiration and 10/30 (33%) per transfer]. No pregnancies were obtained in the group without a ≥80% increase in inhibin A (Table IV).

Discussion

The maturation, fertilization and cleavage rates observed in the present experiments are consistent with findings in previous studies. We observed, however, a higher pregnancy rate and a higher implantation rate, indicating improved embryo development of the oocytes. Previous studies have reported pregnancy rates of 2% when up to three embryos are transferred (Barnes et al., 1996; Trounson et al., 1998). Some centres have transferred more embryos resulting in higher pregnancy rates (Cha et al., 2000), but the implantation rate...
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Table IV. The maturation, fertilization and cleavage rates of oocytes obtained after an increase in both oestradiol (≥100%) and inhibin A (≥80%) (n = 83 cycles).

<table>
<thead>
<tr>
<th>No. aspirations</th>
<th>No. oocytes for IVM</th>
<th>MII (%)</th>
<th>2PN (%MII)</th>
<th>Cleavage (%MII)</th>
<th>Pregnancies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥80% increase</td>
<td>42</td>
<td>191</td>
<td>116 (61)</td>
<td>94 (81)</td>
<td>81 (70)</td>
</tr>
<tr>
<td>&lt;80% increase</td>
<td>41</td>
<td>182</td>
<td>112 (62)</td>
<td>78 (70)</td>
<td>70 (63)</td>
</tr>
</tbody>
</table>

IVM = in-vitro maturation; MII = metaphase II; 2PN = fertilization with two pronuclei.

*P < 0.02 (Fisher’s exact test).

of embryos derived from IVM oocytes has been low, compared with the results from conventional IVF.

It has been questioned whether inadequate or incomplete cytoplasmic maturation of oocytes leading to developmentally incompetent embryos contributes to the high incidence of pregnancy failure in previous studies. A number of factors may account for this, including suboptimal culture conditions during IVM or suboptimal quality of the oocytes (Moor et al., 1998) due to the age of the women, endocrinological disturbance or previously failed IVF (Barnes et al., 1996; Russell et al., 1997; Trounson et al., 1998). While some studies have focused on improved culture media (Hwu et al., 1998; Trounson et al., 1998), others have tried to optimize the quality of the oocyte (Russell et al., 1997; Trounson et al., 1998; Wynn et al., 1998; Chian et al., 2000; Suikkari et al., 2000).

We intended to obtain oocytes with expected good quality and therefore we focused on regularly menstruating women referred for IVF due to male factor infertility and/or tubal disease. In a previous study (Wynn et al., 1998), an increased maturation rate of oocytes after pre-treatment with FSH was found. However, in our first experiment, we did not find that FSH priming of the women before oocyte retrieval improved the maturation rate in vitro. There are two possible explanations for the difference observed: (i) the culture conditions used or (ii) the timing of aspiration. Wynn et al. (1998) used a defined serum-free medium, while we supplemented our culture medium with serum from the patient. The timing of aspiration differed, as Wynn et al. performed the aspiration on a fixed day (day 7 in the menstrual cycle), while the day of aspiration in the present study was monitored by ultrasound, and oocyte collection was performed the day after a leading follicle of 10 mm in diameter was demonstrated.

The oocytes were not fertilized in the study of Wynn et al. In our study, FSH priming had no effect on fertilization or cleavage rates. This is consistent with previous studies (Trounson et al., 1998). We know from other studies that a wide inter-individual variation in the concentrations of FSH may reflect differences in the FSH threshold and differences in ovarian sensitivity to FSH (Schipper et al., 1998). This may be one of the possible reasons for the lack of difference in maturation rates and fertilization rates, although the concentrations of FSH differ.

Previous studies in humans have shown that 80% of immature oocytes show nuclear maturation (extrusion of a polar body) and will be at metaphase II by 48–54 h of culture (Trounson et al., 1994; Russell et al., 1997). Most of the oocytes inseminated after such an interval have been at metaphase II arrest for 20–30 h, which places them well past the optimal fertilization time and may compromise their developmental competence. Considerable asynchrony of maturation has been observed and by 24 h of maturation a number of metaphase II oocytes can be obtained. Furthermore, those oocytes reaching metaphase II first seemed to be the most competent to develop into blastocysts (Barnes et al., 1996) and the implantation potential of the embryos may also be improved by maturation for 36 h compared with 48 h (Mikkelsen et al., 1999). The 28 h IVM period had a significant benefit in that it allowed the insemination to be performed during working hours; inseminations had to be performed at night when the 36 h IVM schedule was used.

It appeared from experiment 2 that early insemination can be used with relative efficacy. We
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found no significant differences in the maturation, fertilization or pregnancy rates of oocytes which had been matured for 28 h compared with 36 h. The use of early insemination may prevent oocytes from becoming senescent before they are inseminated; however, the optimal time of insemination has not yet been established.

The importance of the timing of oocyte retrieval in human IVM is presently unclear. The maturation potential of the oocytes may be affected by dynamic physiological changes which occur within the follicle, and these factors may change from the early follicular phase before selection of a dominant follicle to the late follicular phase after selection of a dominant follicle (Fauser and van Heusden, 1997).

It has been considered that the dominant follicle can be recognized by ultrasound when its diameter has reached 10 mm (Pache et al., 1990; Fauser and van Heusden, 1997). In the present study, we aimed for oocyte collection to coincide with selection of the dominant follicle and we were able to obtain a clinical pregnancy rate of 12.6% per aspiration and 17.4% per transfer.

Growth and development of the dominant follicle are correlated with secretion of both oestradiol (van Dessel et al., 1995) and inhibin A into the circulation. The increase in serum concentration of inhibin A has been found to parallel that of oestradiol, but with a delay of 1–2 days (Schipper et al., 1998). In humans, a linear relationship between follicular fluid concentrations of inhibin A and follicular size and maturity has been reported, therefore the increase in serum inhibin A seems to give evidence of growth of the dominant follicle. In the present study, pregnancies were only obtained in cycles where a substantial increase in both oestradiol (≥100%) and inhibin A (≥80%) was observed, whereas none were obtained in cycles without such increases in these hormones. This suggests an improved cytoplasmic maturation of oocytes obtained from follicles at a more advanced stage of folliculogenesis.

As well as gonadotrophins and oestradiol, our medium for maturation contained serum from the patient, obtained on the day of aspiration. This means that other hormones and growth factors may have played a role in the process of IVM. Studies in cattle suggest that inhibin A may play an important role during the final stages of oogenesis. Recent reports have indicated that both inhibin A and activin A may enhance cytoplasmic maturation, so these hormones or their combination in the serum may potentially have improved the embryonic developmental competence of the oocytes (Alak et al., 1999).

Follicle size and the serum concentration of oestradiol have long been used as indicators of oocyte maturity in ovulation induction and conventional IVF. Both these parameters indicate the fertilization potential of oocytes in IVF cycles. We found in the present study that the same parameters may play a role in decision-making before aspiration in IVM. Furthermore, monitoring the concentration of inhibin A seemed to be of added value in assessing the optimal time for oocyte retrieval as a clinical pregnancy rate of 24% per aspiration was obtained in 41 cycles with a substantial increase in both hormone values. These data may contribute to improving clinical procedures to obtain immature oocytes, leading to an attractive alternative to ovarian stimulation for IVF. As well as the clinical benefits of lowering side-effects, this treatment may reduce the costs of IVF especially since no benefit of low dose FSH priming (compared with the natural cycle) was demonstrated. In IVF/ICSI undertaken because of male factor infertility, most of the women are producing their own FSH to assist the stimulation of ovarian follicles. In these naturally cycling women, the timing of aspiration may be crucial in IVM.

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References
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