Time interval between FSH priming and aspiration of immature human oocytes for in-vitro maturation: a prospective randomized study

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Abstract

This prospective randomized controlled study was performed to examine the influence of coasting for 2 days versus 3 days following a fixed daily dose of FSH for 3 days. The outcome was 2-fold. In the first experiment (n = 50 cycles), the incidence of apoptosis in granulosa cells was compared. In the second experiment (n = 28 cycles), the rates of maturation, fertilization, cleavage, pregnancy and implantation were compared. In addition, clinical pregnancy rate per aspiration was registered. Granulosa cells were collected from follicular aspirates and pooled for each patient. The APOPTAG® Detection Kit was used for staining of the granulosa cells and detection of apoptosis. Oocytes were matured in vitro for 28–30 h before intracytoplasmic sperm injection. The incidence of apoptosis in granulosa cells did not differ between granulosa cells obtained after 2 days coasting (n = 25 cycles) compared with granulosa cells obtained after 3 days coasting (n = 25 cycles) (26.2 versus 26.2%). When oocytes obtained after coasting for 2 days (n = 12 cycles) were compared with oocytes obtained after coasting for 3 days (n = 16 cycles), no significant difference was found between rates of maturation (63 versus 65%), fertilization (60 versus 68%), cleavage (86 versus 92%) or implantation [5/12; 42 versus 21/12 (8%)]. A higher clinical pregnancy rate per aspiration [5/16 (31%) versus 1/12 (8%)] was obtained after coasting for 3 days compared with coasting for 2 days. The difference was not significant. This randomized study showed no difference in apoptosis of granulosa cells and no difference in developmental competence of oocytes obtained after coasting for 3 days compared with 2 days coasting.

Keywords: coasting, FSH priming, human oocytes, in-vitro maturation

Introduction

In recent years, increased efforts have been devoted to in-vitro maturation (IVM) of human oocytes (Cha et al., 2000; Trounson et al., 2001). Data regarding the benefit of in-vivo FSH priming before aspiration are conflicting. Some studies have found an improvement in the harvest of oocytes and improved oocyte quality after priming with FSH prior to aspiration (Wynn et al., 1998; Suikkari et al., 2000), while others were unable to detect this (Mikkelsen et al., 1999). The studies differed in stimulation regimens and timing of aspiration. Experiments in cattle have led to the hypothesis that competent oocytes require a plateau phase of follicular growth to express full developmental capacity (Sirard and Blondin, 1996). Moderate follicle stimulation with gonadotrophins followed by a gonadotrophin-free period to induce a reduced growth rate has been proposed, as increased oocyte competence was obtained when oocytes were aspirated 48 h, but not 24 or 72 h, after the bolus FSH injection (Blondin et al., 1997; Sirard et al., 1999). Withholding FSH before aspiration has been suggested as a treatment modality in humans (Suikkari et al., 2000); however, the duration of the coasting period was not established.

Apoptosis in the granulosa cells and cumulus cells is associated with follicular atresia occurring during natural (Yuan and Giudice, 1997) and stimulated ovarian cycles (Nakahara et al., 1997a,b, Oosterhuis et al., 1998; Høst et al.,...
Little information about the importance and necessity of apoptosis in granulosa cells of immature human follicles is available. To date, so far as is known, no prospective study has been primarily designed to investigate the period of coasting. The need for such studies has been stressed. To that end, a prospective controlled study has been performed to examine the influence of coasting of FSH for 2 versus 3 days following a daily fixed dose of recombinant FSH (rec-FSH) for 3 days. The outcome was 2-fold. In the first experiment, the incidence of apoptosis in granulosa cells was compared. In the second experiment, the rates of maturation, fertilization, cleavage and implantation were compared. In addition, the clinical pregnancy rate per aspiration was registered.

Materials and methods

Subjects

All patients were regularly cycling women recruited from patients referred for IVF/intracytoplasmic sperm injection (ICSI) due to male factor infertility and/or tubal factor infertility. All the women were <38 years of age with a body mass index (BMI) between 18 and 29 kg/m². The basal FSH concentration was <15 IU/l (Scott et al., 1989) and the basal inhibin B level >45 pg/ml (Seifer et al., 1997). Patients with infertility caused by endocrine abnormalities such as hyperprolactinaemia and patients with one ovary or ovarian endometriosis were excluded. Patients with possible poor quality of the oocytes, i.e. patients with a low (<20%) cleavage rate after ovarian stimulation and IVF or ICSI, were also excluded, as were patients who had had more than three previous failures to conceive with IVF/ICSI. The IVM procedure was approved by the local ethics committee, and written informed consent was obtained from all participants.

An ultrasound examination was performed on day 3 and the cycle was cancelled if an ovarian cyst(s) >10 mm in diameter was observed. On day 3, the women began priming with rec-FSH (Gonal-F; Serono, Geneva, Switzerland) 150 IU per day for 3 days. The patients were assigned randomly to two groups with the use of an envelope with random numbers. Randomization was performed before stimulation was initiated. In one group aspiration was performed on day 8 after coasting for 2 days, and in the other group aspiration was performed on day 9 after coasting for 3 days.

Experiment 1

Granulosa cells from the aspirated follicles were collected and pooled for each cycle. For each cycle, the number of oocytes retrieved, the number of atretic oocytes and the incidence of apoptosis in granulosa cells were recorded. The incidence of apoptosis in granulosa cells was compared between group Ia (follicles aspirated after coasting for 2 days, n = 25 cycles) and group Ib (follicles aspirated after coasting for 3 days, n = 25 cycles).

Granulosa cell preparation and assessment of apoptosis

The granulosa cells were collected from the follicular aspirates during aspiration and washed twice in Earle’s balanced salt solution (EBSS; Medi-Cult, Denmark). The pellet with granulosa cells was pipetted onto a Silan-coated microscope slide and a thin smear was prepared. Slides were air-dried and fixed in 96% (v/v) ethanol for 5 min. An APOPTAG® in-situ apoptosis detection kit – peroxidase (INTERGEN Oxford, catalog no: S7100-KIT) was used, with some modifications for staining of the granulosa cells for the detection of apoptosis (Høst et al., 1999). The method is designed to label the free 3´-OH DNA termini in situ with chemically labelled and unlabelled nucleotides. The nucleotides contained in the reaction buffer are enzymatically added to the DNA by terminal deoxynucleotidyl transferase (TdT). TdT catalyses a template-independent addition of nucleotide triphosphates to the 3´-OH ends of double or single-stranded DNA. The incorporated nucleotides from an oligomer composed of digoxigenin are allowed to bind an anti-digoxigenin antibody that is conjugated to peroxidase. The localized peroxidase enzyme then catalytically generates an intense signal from

Figure 1. Micrograph showing granulosa cells with apoptosis (brown coloured) and granulosa cells without apoptosis (green coloured). Scale bar = 100 µm.
chromatin. For negative-stained control, water instead of TdT enzyme mixed into reaction buffer was used. Background staining was found to be <3%.

Apoptosis and morphology were scored according to peroxidase/DAB staining. To evaluate the degree of apoptosis, about 300 granulosa cells were analysed on each slide, if possible, under the microscope by oil immersion at ×1000 magnification and bright light field illumination (Figure 1) (Mikkelsen et al., 2001a).

The slides were assessed by the same observer twice, as they were randomly reassessed. The intra-observation variation was found to be <2%.

The incidence of apoptosis was examined on a per patient basis. The apoptotic index is the degree (%) of apoptotic, activated granulosa cells per cycle.

Experiment 2

Oocytes were matured for 28–30 h before insemination with ICSI. Oocytes obtained after coasting for 2 days (n = 12 cycles) and oocytes obtained after coasting for 3 days (n = 16 cycles) were compared with respect to rates of maturation, fertilization, cleavage and implantation. In addition, clinical pregnancy rates were compared. It was not possible in this experiment to examine whether rates of maturation, fertilization, cleavage or pregnancy are correlated with the incidence of apoptosis of the corresponding granulosa cells, as the granulosa cells were pooled and examined on a per patient basis.

Only oocytes that were classified as having a multilayer or sparse cumulus were used. The oocytes were matured in ‘Dyrkningsmedie til IVM’ (Medi-Cult), rec-FSH 0.075 IU/ml (Gonal-F; Serono), HCG 0.5 IU/ml (Profasi; Serono) and 10% ‘Dyrkningsmedie til IVM’ (Medi-Cult), rec-FSH 0.075 IU/ml (Gonal-F; Serono), HCG 0.5 IU/ml (Profasi; Serono) and 10% serum (v/v) from the patient (Mikkelsen et al., 2001b). Oocytes were matured in 25 µl drops of IVM medium under paraffin oil for 28–30 h at 37°C in a humidified atmosphere of 5% CO₂ in air.

The oocytes were inseminated by ICSI. This enabled microscopic confirmation of the presence of the first polar body, which was the criterion used to classify oocytes as matured to metaphase II. However, extrusion of the first polar body is not an adequate indicator of the completion of oocyte maturation, because it is indicative only of nuclear maturation. Evaluation of the developmental capacity of oocytes following fertilization (clinical pregnancy rate) is the most useful way to assess cytoplasmic maturation.

The technique of ICSI was similar to that described previously (Smith et al., 2000), but in brief, the cumulus cells were removed using hyaluronidase, 80 IU/ml (Medi-Cult), and a single spermatozoon was immobilized in polyvinylpyrrolidone (Medi-Cult). Oocytes that appeared atretic (dark and shrunken) or immature (germinal vesicle oocytes) after maturation were discarded.

After ICSI, the oocytes were cultured individually in 25 µl droplets of IVF medium (Medi-Cult) in Falcon petri dishes (Life Technologies) under oil 5% CO₂ and humidified air at 37°C. After insemination, embryos were cultured for 3 days, at which time suitable embryos (maximum 2) were transferred.

Endometrial priming consisting of 17-β-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 was initiated and continued until the pregnancy test. Oestradiol and progesterone were continued if the pregnancy test was positive, until 50 days gestation.

Ultrasound examination

Ultrasound examination was performed on day 3, days 6–7 and daily or every second day until the day of aspiration using a 7.5 MHz transvaginal transducer (B and K Medical, Gentofte, Denmark). The follicular diameter was calculated as the mean of the longest follicular axis and the axis perpendicular to it. A clinical pregnancy was defined as evidence of intrauterine fetal heart activity 3 weeks after embryo transfer visualized by ultrasonography.

Aspiration, maturation, fertilization and embryo culture

This has been described in detail previously (Smith et al., 2000; Mikkelsen et al., 2001a). In brief, aspiration was performed transvaginally with a 17G single lumen needle (K-OPSC-1225; Cook, Queensland, Australia) connected to a syringe to induce the aspiration vacuum and the follicular aspirates were transferred into tubes containing Ham’s F-10 medium with heparin at 37°C (Life Technologies, Roedovre, Denmark). The aspirates were filtered (Falcon 1060; 70 µm mesh size) to remove erythrocytes and small cellular debris. The oocytes were isolated under a stereomicroscope and washed twice. The oocytes and/or their cumulus investments were classified as follows: multilayered cumulus, sparse cumulus, nude or atretic.

Statistics

Statistical analyses were performed using chi-squared test or Fisher’s exact test when appropriate. Mann–Whitney rank sum test was calculated and used for the comparison between apoptosis in the granulosa cells. Values were considered statistically different when a p-value was <0.05.

Table 1. Incidence of apoptosis in granulosa cells from immature FSH primed follicles after deprivation for 2 days (group Ia) or 3 days (group Ib) before aspiration.

<table>
<thead>
<tr>
<th></th>
<th>Group Ia</th>
<th>Group Ib</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. cycles</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Apoptosis median (%)</td>
<td>26.2²</td>
<td>26.2²</td>
</tr>
<tr>
<td>Range (%)</td>
<td>5.9–65</td>
<td>7.3–56.3</td>
</tr>
<tr>
<td>No. oocytes</td>
<td>142</td>
<td>124</td>
</tr>
<tr>
<td>No. atretic oocytes</td>
<td>57 (40)</td>
<td>50 (40)</td>
</tr>
</tbody>
</table>

Values in parentheses are percentages.
²Analysed by Mann–Whitney. There was no statistical difference between the groups.
The studies differed in stimulation regimens and timing of aspiration. While Wynn et al. (1998) performed the aspiration on a fixed day (the day after the last FSH injection), Mikkelsen et al. (1999) performed the aspiration with a delay after the last FSH injection. The oocytes in the study by Wynn et al. (1998) were not inseminated and therefore no information about developmental capacity after fertilization can be obtained from that study.

Resumption of meiosis is not itself a sufficient guide to developmental capacity (Eppig et al., 1994). Even though the oocytes may be competent to complete nuclear maturation, they may be deficient in cytoplasmic maturation; most deficiencies in oocytes during in-vitro maturation are associated with cytoplasmic reprogramming for reasons that remain unknown (Fulka et al., 1998).

IVM of aspirated immature oocytes requires a period of 24–48 h in culture. Differences in embryonic development between oocytes matured after 24 h compared versus oocytes matured after 48 h have been described (Chian and Tan, 2002). In the present study, the majority of oocytes were matured after 28–30 h.

Experiments in cattle have led to the hypothesis that competent oocytes require a plateau phase of follicular growth to express full developmental capacity (Sirard and Blondin, 1996). Moderate follicle stimulation with gonadotrophins followed by a gonadotrophin-free period to induce a reduced growth rate has been proposed. Increased oocyte competence was obtained when oocytes were collected 48 h but not 24 or 72 h after the bolus FSH injection (Blondin et al., 1997; Sirard et al., 1999). Similar studies in humans are lacking, although the need for such studies has been stressed.

A longer interval between FSH administration and aspiration would allow early atresia to take place in the granulosa cells, and this might enhance developmental competence of the oocytes. There is little information in humans with regard to the importance and necessity of apoptosis in granulosa cells of immature follicles. Høst et al. (2000) have reported a significantly higher degree of apoptosis in cumulus cells from immature oocytes compared with cumulus cells from MII oocytes, when oocytes were obtained after ovarian stimulation. However, the immature oocytes in that study (Høst et al., 2000) may represent an inferior population, as they failed to mature although the follicles were exposed to supra-physiological concentrations of gonadotrophins, and maturation in vitro of these immature oocytes has demonstrated that developmental competence is markedly inferior to that of their in-vivo matured counterparts (Kim et al., 2000).

In the present study, the incidence of apoptosis of granulosa cells originating from follicles deprived for FSH for 3 days did not differ from the incidence of apoptosis of granulosa cells originating from follicles deprived for FSH for 2 days. Furthermore, the incidence of apoptosis did not differ between granulosa cells originating from small follicles compared with granulosa cells originating from larger follicles. However, the data in the present study are measured on a per patient basis and

<p>| Group Ia (n = 12 cycles): FSH deprivation for 2 days | Values in parentheses are percentages. |
| Group Ib (n = 16 cycles): FSH deprivation for 3 days |</p>
<table>
<thead>
<tr>
<th>No. oocytes</th>
<th>No. oocytes for IVM</th>
<th>MII</th>
<th>2PN</th>
<th>Cleavage</th>
<th>Implantations</th>
<th>Pregnancies</th>
</tr>
</thead>
<tbody>
<tr>
<td>53</td>
<td>29 (55)</td>
<td>19 (66)</td>
<td>13 (68)</td>
<td>12 (63)</td>
<td>1/12 (8)</td>
<td>1 (8)</td>
</tr>
<tr>
<td>75</td>
<td>45 (60)</td>
<td>28 (62)</td>
<td>15 (53)</td>
<td>12 (43)</td>
<td>5/12 (42)</td>
<td>5 (31)</td>
</tr>
</tbody>
</table>

| Table 1. Maturation, fertilization, cleavage, implantation and pregnancy rates of oocytes obtained after deprivation of FSH for 2 days versus oocytes obtained after deprivation of FSH for 3 days after FSH priming. There were no statistically significant differences between the groups. |

<table>
<thead>
<tr>
<th>Table 2. Maturation, fertilization, cleavage, implantation and pregnancy rates of oocytes obtained after deprivation of FSH for 2 days versus oocytes obtained after deprivation of FSH for 3 days after FSH priming. There were no statistically significant differences between the groups.</th>
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<td>1 (8)</td>
</tr>
<tr>
<td>Group Ib (n = 16 cycles): FSH deprivation for 3 days</td>
<td>75</td>
<td>45 (60)</td>
<td>28 (62)</td>
<td>15 (53)</td>
<td>12 (43)</td>
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</table>
the incidence of granulosa cell apoptosis on a per ovary basis cannot be associated directly with each follicle.

Development to a live fetus following fertilization is generally considered to be a valid criterion for developmental capacity of the oocyte (Eppig et al., 2002). Until now, there has been no other agreed assay for the completion of oocyte maturation. In the future, non-invasive spindle imaging may prove useful in evaluation of oocyte quality (Eichenlaub-Ritter, 2002). In the present study, no difference in rates of maturation, fertilization, cleavage or implantation was observed. An increased clinical pregnancy rate was found after coating for 3 days compared with coating for 3 days. The difference did not reach significance, probably due to the small number of patients. However, as in other reproductive studies with human oocytes, this study was limited by legal and ethical restrictions.

In conclusion, this randomized study showed no difference in apoptosis of granulosa cells and no difference in developmental competence of oocytes obtained after 3 days coating compared with 2 days coating.

Acknowledgement

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