Endocrinological and ultrasonographic variations after immature oocyte retrieval in a natural cycle

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BACKGROUND: In-vitro oocyte maturation is an appealing alternative in reproductive medicine but the results obtained are still poor. The aim of our prospective and observational investigation was to study the hormonal modifications that immature follicular aspiration might induce in a natural cycle as well as the implications that these alterations may have in the endometrium. METHODS: Eleven patients (13 cycles) were included in our in-vitro oocyte maturation programme. Ovaries were scanned with transvaginal probes every day and follicular aspiration was performed when a follicle of 9 mm was visualized. Blood was also drawn for hormonal analysis. Endometrial thickness was recorded every day after oocyte retrieval. Two endometrial biopsies were taken on days 6 and 8 after oocyte retrieval. RESULTS: We observed a significant drop in serum oestradiol concentrations after immature oocyte retrieval previous to follicle dominance. Immediately after, rises in both FSH and LH were detected. Also, a new dominant follicle started to grow 3–4 days later. Steroid hormones secreted by this newly recruited follicle were significantly lower than in controls, inducing inadequate endometrial thickness. CONCLUSIONS: These studies show that exogenous hormonal administration might be necessary to achieve a correct endometrial growth when in-vitro oocyte maturation is employed in IVF.

Key words: endometrial pattern/hormonal profile/in-vitro oocyte maturation

Introduction

Although the first IVF pregnancy was achieved under a natural cycle (Steptoe and Edwards, 1978), very soon after, controlled ovarian hyperstimulation was implemented as higher pregnancy rates were obtained (Laufer et al., 1983; Pellicer et al., 1989). Together with the advantages of gonadotrophin usage came its complications, such as ovarian hyperstimulation syndrome, multiple pregnancies or the unknown long-term consequences (Bergh and Navot, 1992; Whittermore et al., 1992). These factors fuelled continued investigation about IVF in unstimulated cycles along with the development of assisted reproduction techniques (Eppig et al., 1992; Pavlok et al., 1992). The group from Norfolk, VA, USA reported the first baby born from in-vitro maturation of immature oocytes (Veeck et al., 1983), and since then, important achievements have been made to improve the low pregnancy rate that this technique yields (Barnes et al., 1995; Cha et al., 2000).

There are two main factors related to the low pregnancy rates reported with these techniques: (i) the incomplete cytoplasmic maturation, which is not parallel to the nuclear maturation observed, which may cause poor embryo quality after oocyte fertilization (Flood et al., 1990; Eppig, 1996), and (ii) an inadequate endometrial priming, which may cause implantation failure (Trounson et al., 1994; Russell et al., 1997).

Several investigations have covered this second issue so as to obtain a correct endometrium prior to embryo transfer. Generally speaking, most groups administer exogenous oestradiol and progesterone during the natural cycle (Trounson et al., 1994) or hormonal replacement therapy is administered in a similar pattern as in an oocyte donation programme (Cha et al., 1991). Russell studied the effect of 17-beta oestradiol administered either at the onset of the follicular phase or at the beginning of the midfollicular phase, finding that the first choice resulted in a lower maturation and fertilization rate, and a lower number of embryos to be transferred (Russell et al., 1997). Therefore, even though a general agreement exists on exogenous oestradiol administration to prepare the endometrium, this might be deleterious depending on when it is started, and no evidence exists on the benefit of this treatment.

The purpose of our investigation was to study the hormonal variations that appear in a natural cycle in which early antral follicles are aspirated before the leading follicle reaches dominance. Patients were endocrinologically followed after follicular puncture, and serum gonadotrophin and steroid
concentrations compared with normal controls. Moreover, an ultrasonographic follow-up was performed to evaluate the endometrial modifications after immature oocyte retrieval and follicular growth. Following oocyte maturation, fertilization and embryo development until blastocyst stage were observed as previously reported (Cobo et al., 1999). In addition, the endometrium was biopsied at the time of the hypothetical embryo implantation and dated so as to ascertain whether early follicular aspiration induced changes that might need supplementation of the patients with exogenous steroids.

Materials and methods
A total of 11 patients (13 cycles) were voluntarily included in our in-vitro oocyte maturation programme with donor oocytes between October 1, 1997 and April 30, 1998. Mean age of the patients was 25.7 ± 3.9 years. All of them had a regular menstrual cycle and had not been under any hormonal treatment in the previous 3 months. Five women (mean age 27.8 ± 2.4 years) with regular menstrual cycles, were included as a control group. Patients with significant gynaecological diseases, including polycystic ovaries, were excluded in both groups. The study was approved by our Institution’s ethics committee, and all couples were required to sign a written informed consent after the provision of complete information.

Starting on the 2nd or 3rd day of the cycle, ovaries were scanned with transvaginal probes daily. The diameter of the dominant follicle was calculated as the mean of the two main diameters measured. If any follicle >10 mm from the previous cycle was detected in the first ultrasound scan, the cycle was cancelled. Follicular aspiration was performed when a follicle of 9 mm was visualized since it has been considered that the dominant follicle can be easily recognized by ultrasound when measuring 10 mm (Fauser and van Heusden, 1997). Blood was also drawn for hormonal analysis. The detailed description of the follicular aspiration methodology has been described in detail elsewhere (Requena et al., 2000).

Endometrial thickness was also recorded every day after oocyte retrieval. This date was assessed measuring the distance from the hyperechogenic interface of the endometrium and the myometrium to the opposite interface including the stronger midline echo (Shoham et al., 1991). Two endometrial biopsies were taken on days 6 and 8 after oocyte retrieval.

The control group followed similar ultrasound examinations in order to evaluate the follicle size and endometrial thickness and pattern. Blood was taken on days 2, 5, 8, 11, 13, 14 and 15 of the menstrual cycle.

Hormone Measurements
The samples were stored at −20°C in aliquots for subsequent oestradiol, FSH, LH, and progesterone analysis. Serum oestradiol, FSH, and LH were analysed using a commercially available microparticle enzyme immunoassay kit (Abbott Laboratories, Abbott Park, IL, USA). Inter- and intra-assay variability for oestradiol was 7.9 and 6.1% respectively, 8.5 and 4.3% for FSH, and 5.7 and 4.1% for LH. Progesterone was analysed using an automated quantitative test combining immunoenzymatic detection with fluorescence (ELFA, BioMérieux, Charbonnières les Bains, France). Inter- and intra-assay variability were both 4.3%.

Endometrial biopsies
Two endometrial biopsies were taken with a Cornier device (Gynecetics, Hamont-Achel, Belgium) in six patients from the study group and kept in 96% alcohol until processed for pathology employing the classical criteria (Noyes et al., 1950).

Statistical Analysis
Data were expressed as means ± SEM. Comparisons between means were performed with the use of the Student’s t-test, after confirming variance homogeneity using Levene’s test. A value of P < 0.05 was considered significant. Statistical calculations were performed using Sigmastat for Windows, version 2.0 (Jandel Scientific Corporation, San Rafael, CA, USA).

Results
Considering the size of the follicle as the criterion for oocyte recovery, the day of oocyte retrieval was named as day P and occurred on day 7.1 ± 1.4 of the cycle. Serum FSH values are shown in Figure 1A. On day P + 1 (day 8 of the cycle), values differed significantly between the study group and controls (10.8 ± 1.0 versus 7.4 ± 1.2 mIU/ml respectively; P < 0.05). Also, on day P + 7 serum FSH levels differed significantly (6.1 ± 0.6 versus 12.2 ± 1.3 mIU/ml; P < 0.05). The pattern of FSH secretion was different between groups, despite the absolute values. In fact, Figure 1A clearly shows
Maturation \textit{in vitro} of human oocytes

**Figure 2.** Serum oestradiol (A) and Progesterone (B) levels. Open circles show values in the study group whereas closed circles show the controls. OPU = Oocyte retrieval. *$P < 0.05$. Values are means $\pm$ SEM.

An increase in FSH after oocyte retrieval which is not seen in the controls.

Similarly, serum LH levels throughout the menstrual cycle in both groups differed on day P + 1 (7.2 $\pm$ 0.5 versus 5.0 $\pm$ 0.6 mIU/ml; $P < 0.05$) (Figure 1B) and on day P + 7 (10.3 $\pm$ 3.1 versus 27.3 $\pm$ 2.8 mIU/ml; $P < 0.05$). This figure again shows an increase in LH in response to follicular aspiration and a mid-cycle LH peak delayed by 3 days in the study group as compared with the controls. The intensity of the LH peak was also qualitatively different when both groups were compared.

Figure 2A represents serum oestradiol concentrations from patients in the study group as compared with controls. Significant differences between groups were observed on days P + 1 (44.6 $\pm$ 5.2 versus 86.3 $\pm$ 19.6 pg/ml, respectively; $P < 0.05$), P + 6 (134.9 $\pm$ 23.4 versus 287.6 $\pm$ 47.4 pg/ml; $P < 0.05$) and P + 7 (158.3 $\pm$ 19.4 versus 235.2 $\pm$ 35.4 pg/ml; $P < 0.05$). An interval of 3 days was observed between the peak of oestradiol in the study group and controls. Also, a significant difference was observed between groups in the secretion of oestradiol.

When serum progesterone was evaluated in both groups, similar values were found, except for day P + 8 (2.4 $\pm$ 1.1 versus 5.4 $\pm$ 2.3 ng/ml; $P < 0.05$) in which ovulation already occurred in the controls, whereas progesterone secretion was still low in the study group (Figure 2B). Serum progesterone was elevated in the study group one day later.

The mean diameter of the dominant follicle was followed in the study group and control patients until ovulation was observed (Figure 3). Values were significantly different on days P + 4 (12.0 $\pm$ 0.8 versus 17.5 $\pm$ 0.6 mm respectively; $P < 0.05$), P + 6 (14.4 $\pm$ 0.9 versus 18.3 $\pm$ 0.7 mm; $P < 0.05$), P + 7 (15.0 $\pm$ 1.0 versus 18.9 $\pm$ 1.2 mm; $P < 0.05$) and P + 8 (16.9 $\pm$ 0.8 versus 19.5 $\pm$ 1.9 mm; $P < 0.05$).

Endometrial thickness was evaluated by transvaginal ultrasound during the menstrual cycle (Figure 4). Significant differences were found most of the days of the study between the study group and the controls: P + 4 (5.4 $\pm$ 0.2 versus 6.6 $\pm$ 0.3 mm respectively; $P < 0.05$), P + 6 (6.5 $\pm$ 0.6 versus 8.7 $\pm$ 0.7 mm; $P < 0.05$), P + 7 (6.7 $\pm$ 0.6 versus 9.8 $\pm$ 0.3 mm; $P < 0.05$) and P + 8 (7.6 $\pm$ 0.4 versus 10.4 $\pm$ 0.3 mm; $P < 0.05$).

Endometrial samples were taken in six patients in the study group and dated. The pathology study of the endometrial biopsies showed that all of them were in a proliferative phase on day P + 6. On day P + 8, one subject was dated as mid-proliferative phase, three as mid- and late-proliferative phase and two as late-proliferative phase. No correlations were found between oestradiol levels and the pathology studies of these samples.

**Discussion**

Although several arguments are in favour of \textit{in-vitro} oocyte maturation obtained from unstimulated cycles, the outcome is still much lower when compared with gonadotrophin-stimulated cycles (Cobo \textit{et al.}, 1999). Thus, our efforts should be directed to investigate the factors involved in two main...
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erfection after immature oocyte fertilization (Flood et al., 1990; Eppig, 1996); and (ii) an inappropriate endometrial priming
which may hamper embryo implantation (Trounson et al., 1994; Russell et al., 1997).
We have focused on the second issue, thus the aim of our investigation was to study the hormonal modifications that immature follicular aspiration might induce in a natural cycle as well as the implications that these alterations may have in endometrial priming for embryo implantation. When gonadotrophin levels were evaluated in peripheral blood, a rise in both FSH and LH was observed after follicle aspiration as compared with controls. One possible explanation for this finding is the fact that oocyte retrieval induced the demise of the dominant follicle, inducing the selection of a new follicle from the same cohort that will need the assistance of the pituitary to grow, as manifested in our study by an increase in the levels of both FSH and LH. These findings confirm early studies performed in women undergoing laparoscopy in whom the corpus luteum was destroyed and a rise was observed in serum gonadotrophin concentrations before dominance (Baird et al., 1984).

The suspicion that in the course of oocyte retrieval the destruction of the follicle could corrupt both endocrine and paracrine signalling was also confirmed by measuring serum oestradiol. We observed a drop in the oestradiol concentrations that induced a positive feed-back, inducing the pituitary release of gonadotrophins.

Based on serum oestradiol and LH concentrations as well as on the ultrasound scans, our studies showed that a new dominant follicle appeared ∼3–4 days after oocyte retrieval, confirming previous studies in monkeys in which the observation of a new dominant follicle was related to the stage of the cycle in which the selected follicle was removed. Goodman and Hodgen showed in primates that 12.4 days were required for a new FSH and LH surge when the dominant follicle was removed (Goodman et al., 1977; Goodman and Hodgen, 1979). These observations were later confirmed in humans (Nilsson et al., 1982). However, this period was reduced to 4 days if the follicle was removed before becoming dominant (Goodman et al., 1982; Hodgen, 1982). Thus, our studies confirm this early work performed in primates and show, for the first time, that if a selected human follicle is aspirated before becoming dominant, a new follicle from the cohort will replace it within 3–4 days.

The next issue to be discussed is whether the quality of this newly recruited follicle is the same and has the same impact on the peripheral target organs, especially the endometrium. Our studies show that the new dominant follicle is not as healthy as that which initially developed and was destroyed by aspiration. In fact serum oestradiol concentrations were significantly lower after oocyte retrieval and the preovulatory peak was delayed and of lower intensity as compared to control non-aspirated women. As a consequence, the endometrial thickness never reached the values obtained in the controls. Also, follicular size was always smaller in this newly recruited follicle than in the controls. Moreover, the LH peak probably also reflects the effect of oestradiol in the hypothalamus. LH surge was observed in 61.5% (8/13) of the cycle in the study group. As we can see in Figure 1B, the LH peak was delayed and also of lower intensity than in the controls. Thus, the problem is not only that folliculogenesis is delayed by 3–4 days, but also this new folliculogenesis seems to be of lower quality.

These observations may make it necessary to administer exogenous oestradiol to overcome the insufficient effect of the lower oestradiol concentrations on the endometrium after immature oocyte retrieval.

The absolute levels of progesterone were basically no different between groups. Figure 2B shows, however, a delay in progesterone secretion in the study group and these values also show that ovulation occurred in this group of women regardless of the quality of the preovulatory peak of oestradiol or LH, or the size of the dominant follicle throughout the cycle. Corpus luteum was observed in six cycles of the study group. The fact that endometrial biopsies were out of phase for the day of the cycle only implies that the entire menstrual cycle had a delay.

Taking together all these findings, we have observed how follicular puncture disrupts the endocrinology of the cycle. The new dominant follicle will most probably be a different follicle in the cohort of recruited follicles which has still not undergone atresia. This folliculogenesis has a delay of 3–4 days as compared with normal follicular dynamics, and also is defective in the sense that oestradiol secretion and follicular size are quantitatively impaired. As a possible consequence, endometrial development is affected. The clinical lesson learned from these morphological findings is that oestradiol and progesterone supplementation may be necessary to increase endometrial thickness induced by immature follicle aspiration.
in order to improve the results obtained with a programme of human oocyte in-vitro maturation.

References


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