The cellular activity of different sized follicles in cycles treated with gonadotrophin-releasing hormone analogue

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The activity of granulosa cells derived from different sized follicles surrounding oocytes of apparently comparable maturity was evaluated in hyperstimulated ovaries. Granulosa cells were obtained from women undergoing gamete intra-Fallopian transfer procedures who had been treated with gonadotrophin-releasing hormone analogue and gonadotrophins. Only follicles with oocytes of apparently comparable maturity were considered. Granulosa cells from large and small follicles (≥18 and <15 mm diameter respectively) collected from each patient were cultured separately for up to 48 h in the presence or absence of follicle-stimulating hormone (FSH; 50 ng/ml) or insulin (at varying doses, 0.005–25 μg/ml). We found that aromatase activity was elicited by FSH plus insulin, but not by FSH alone, in granulosa cells from both large and small follicles. Progesterone production was maximal in granulosa cells from large follicles, and in these cells was insensitive to further stimuli, in contrast with those collected from small follicles. Prostaglandin oestradiol was secreted in large amounts by granulosa cells from large follicles. Cyclic adenosine monophosphate (cAMP) concentration did not differ between cells from large and small follicles. Our data demonstrate that there are significant differences in granulosa cells derived from different sized follicles with oocytes of apparently comparable maturity.

Key words: follicle size/granulosa cells/oocyte maturity

Introduction

The use of ovulation induction for multiple follicular growth has introduced the problem of follicular asynchrony (Pellicer et al., 1987). In the hyperstimulated ovary there are a number of developing follicles of varying size and maturity. In this context, clinical management takes into account two available parameters, namely oestradiol plasma concentration and the number of large follicles according to ultrasonography. However, although a direct relationship between follicular size and oocyte fertilizability has been demonstrated (Nayudu et al., 1989), one cannot be sure that oocytes originating from smaller follicles are not able to develop.

The introduction of gonadotrophin-releasing hormone analogue (GnRHa) in assisted reproduction has led to the possibility of the induction of follicular growth without the risk of an undesired luteinizing hormone (LH) surge, thereby reducing the rate of cancelled cycles before follicle puncture compared with non-GnRHa cycles (Testart et al., 1993).

In GnRHa cycles some discrepancies exist between the total number of follicles and the level of peripheral oestradiol, suggesting a possible contribution to oestradiol excretion from small follicles (Testart and Amiel, 1991). In these cycles (Pellicer et al., 1987), the number of mature oocytes recovered was more closely related to the number of small rather than large follicles. However, the proportion of fertilized oocytes significantly decreased when the number of recovered oocytes increased (Testart et al., 1993). Overall, these results have suggested that, while GnRHa treatment can result in many large as well as small follicles with oocytes of apparently comparable maturity, the recruitment of a great number of ovarian follicles is not followed by the production of an equally large number of viable oocytes. It may be assumed that oocytes with poor fertilization ability come mostly from the cohort of small follicles (Testart et al., 1993).

In many studies several parameters, such as steroid production (Botero-Ruiz et al., 1984), aromatase activity (Polan et al., 1984), prostaglandin production (Smith et al., 1991) or cyclic adenosine monophosphate (cAMP) release (Tornell et al., 1991), have been studied in normal and stimulated cycles, taking into consideration different stages of oocyte maturity and varying clinical protocols (Testart et al., 1989). There are very few data investigating the interrelationship between granulosa cell activity and oocyte quality in comparable follicles (as determined by same oocyte maturity). In the light of this, we aimed to study the activity of granulosa cells harvested from different sized follicles with oocytes of apparent comparable maturity.

Materials and methods

Patients

Granulosa cells were obtained from 12 patients undergoing oocyte retrieval for gamete intra-Fallopian transfer (GIFT) treatment. All patients were <39 years of age (range 29–38) with normal ovulatory cycles. The ovarian stimulation protocol consisted of a long-acting GnRHa (leuprolide acetate, one ampoule of 3.75 mg i.m.; Enantone, Takeda, Catania, Italy) administered in the late luteal phase. Once ovarian inhibition was obtained (oestradiol plasma concentrations

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<20 pg/ml), FSH and LH (Metrodin, 75 IU of FSH/ampoule and Pergoovre, 150 IU of FSH and 70 IU of LH/ampoule; both from Serono, Rome, Italy) were administered daily (for a total dose of 2302 ± 523 IU of FSH and 480 ± 350 IU of LH); the duration of therapy was 11.5 ± 1.3 days. Human chorionic gonadotrophin (HCG, Profasi, 5000 IU; Serono) was injected i.m. 36 h prior to retrieval when the oestradiol concentration was >900 pg/ml and at least two follicles reached a diameter ≥16 mm (Table I).

Individual follicular diameters were measured by transvaginal ultrasonography, using a Toshiba apparatus equipped with 5 MHz transducer, and determined to be the mean of the two largest diameters, choosing the scanning plane in which follicles appeared as round as possible in shape. A maximal 20% difference between the two diameters was accepted as a reliable criterion of follicular measurement. Ultrasound measurements were performed by the same operator. The oocytes were scored according to Veeck (1986); under a dissecting microscope pre-ovulatory oocytes showed an expanded corona radiata and cumulus mass associated with large follicular membrane granulosa cells. Only the oocytes with a clear first polar body as seen under the inverted microscope were considered in this study.

The follicles were classified as 'small' (with a diameter <15 mm, i.e. between 13 and 15 mm) and 'large' (with a diameter ≥18 mm, i.e. between 18 and 23 mm).

**Hormones and reagents**

McCoy's 5a medium and Dulbecco's modified Eagle's (DME) medium were obtained from Flow Laboratories (Irvine, Scotland, UK). Percoll was purchased from Pharmacia LKB (Uppsala, Sweden). Insulin from bovine pancreas and astrodendenedione (4-astrogenen-3,17-dione) were purchased from Sigma (St. Louis, MO, USA). Human FSH (Metrodin) from Serono was used for the cultures. According to the Serono guidelines, 1 mg of Metrodin corresponds to 120 IU of FSH.

[1β-3H(N)]Androstenedione (androst-4-ene-3,17-dione; 1 mCi/ml) and [1,2,6,7-3H(N)]progesterone (111.1 Ci/mmol) were obtained from Du Pont–New England Nuclear (Boston, MA, USA).

**Cell culture**

Granulosa cells, aspired according to follicular size (≥18 and <15 mm), were obtained as previously described (Erickson and Magoffin, 1989). Each follicle was measured by ultrasonography and then aspirated in a single tube. The apparatus was than cleaned with McCoy's medium, and all procedures were performed separately in order to avoid any possible contamination. Following isolation, individual cultures of granulosa cells from large and small follicles, harvested from the same patient, were set up. Granulosa cells from aspirated follicles were separated from red blood cells by Percoll gradient centrifugation (300 g for 30 min) into 5 ml columns of 50% Percoll and 50% McCoy's 5a medium. Cells were then washed, resuspended in DME medium and counted by exclusion of Trypan Blue dye. Cell viability was >85% in each isolation. No differences were found in cell viability between granulosa cells from small and large follicles. Sterile graduated conical tubes (Falcon, Oxnard, CA, USA) were used for the collection and isolation of granulosa cells. For each experiment, cells were seeded at a density of 100 000 viable cells/dish in triplicate in 24-well tissue culture plates (Nunc, Copenhagen, Denmark). Results were complete for only 10 out of 12 experiments performed. The number of granulosa cells from each patient totalled 5.6×10⁶ ± 5.2×10⁶ for large follicles and 3.8×10⁶ ± 2×10⁶ for the small ones. Cells were incubated in serum-free DME medium (1 ml/dish) at 37°C in a humidified atmosphere of 5% CO₂ in air for 48 h.

As a control, granulosa cells were cultured for up to 48 h without treatment, or with FSH (50 ng/ml) or insulin (0.5–25 mg/ml) alone, and with FSH plus increasing doses of insulin (from 0.005 to 25 mg/ml) (Andreati et al., 1994).

**Assays**

Progesterone content of the medium was determined using an anti-serum developed in rabbits against progesterone-11BSA (Sigma Immunochemicals, St Louis, MO, USA). The cross-reactivity of this antiserum with 20α-hydroxyprogesterone-3,4-pregnen-3-one is 0.1%; with 20β-hydroxy-4-pregnen-3-one, 4.6%; 3β-hydroxy-3-pregnen-20-one, 22%; 5α-pregnen-3,20-dione, 1.8%; corticosterone, <0.1%; 17β-oestradiol, <0.1%; 17-hydroxyprogesterone, 2.7%; 11α-hydroxyprogesterone, 11.9%; 17β-hydroxyprogesterone, <0.1%. Its sensitivity has been found to be 5 pg progesterone/tube. The intra- and interassay coefficients of variation were 5.5 and 8.2% respectively.

Protein assay was performed following Bradford's (1976) method. Briefly, after aspiration of the medium, 0.1 N NaOH (500 µl in each well) was added. The wells were heated for 30 min at 50°C. The contents of each well were then resuspended with a Pasteur pipette and the suspension, containing cellular material, was tested and read by spectrophotometer (DU 640; Beckman, Palo Alto, CA, USA); no differences were found between treatments and between type of follicles (data not shown).

Aromatase activity was assessed from the stereo-specific release of tritium from [1β-3H]androstenedione to produce 3H₂O (Gore-Langton and Dorrington, 1980). Briefly, granulosa cells (100 000 viable cells/dish) were cultured in serum-free conditions in the absence of androstenedione for 48 h. A triplicate set of dishes without cells was also set up. At 48 h, 10⁻² M cold androstenedione and 100 000 cpm of [3H]androstenedione were added to all dishes; after 8 h incubation, collected media were extracted with 5 ml of chloroform, and the aqueous phase was further subjected to treatment with an MCX column. The radioactivity in the eluate was determined by liquid scintillation counting. The results were complete for only 20 out of 23 experiments performed. No differences were found with the control or with the androstenedione addition. Protein assay was performed following Bradford's (1976) method.
(Boston, MA, USA). The sensitivity was 8 pg/tube for prostaglandin E₂ and <0.2 pmol/ml for cAMP. The inter- and intra-assay coefficients of variation were 5% and 7.5% for prostaglandin E₂ and 4.5% and 9% for cAMP respectively.

Samples of the same patients from the same experiment (culture media from granulosa cells of small and large follicles) were run in the same assay.

**Statistical analysis**

Results were analysed by Student’s paired and unpaired t-test; \( P < 0.05 \) values were considered significant.

**Results**

**Clinical profile of patients**

The clinical features of patients are shown in Table I. The oestradiol peak was correlated with the total number of follicles and the number of small follicles (<15 mm), and not with the number of large follicles (≥18 mm). No linear correlation was found between total units of FSH or LH administered and number of large or small follicles.

**Aromatase activity**

In order to test the responsiveness to FSH and insulin for induction of aromatase activity, cells were treated with FSH (50 ng/ml) alone, insulin (0.5 and 25 µg/ml) alone, or FSH (50 ng/ml) plus increasing doses of insulin (0.005–25 µg/ml) (Figure 1).

In granulosa cells harvested from large follicles (≥18 mm), FSH (50 ng/ml) by itself did not stimulate aromatase activity. In contrast, treatment with insulin (25 µg/ml) alone provoked a marked increase in terms of aromatase activity compared with control values; no significant effect was observed after treatment with a lower dose (0.5 µg/ml). Concurrent treatment with FSH (50 ng/ml) and increasing doses of insulin (0.005–25 µg/ml) revealed that even a small dose of insulin (0.5 µg/ml) in association with FSH was effective in stimulating aromatase activity. Bearing in mind that lower doses of insulin (0.005–0.05 µg/ml) did not show any significant effect on FSH activity (Figure 1A), a combined effect of FSH and insulin can be suggested.

Granulosa cells collected from small follicles (<15 mm) displayed similar behaviour. As already observed in granulosa cells from large follicles, only high doses of insulin (25 µg/ml) were effective in stimulating aromatase activity, and no significant effect was seen after treatment with a lower dose (0.5 µg/ml). Moreover, treatment with FSH (50 ng/ml) plus increasing doses of insulin (0.005–25 µg/ml) elicited a similar effect on aromatase activity to that seen in granulosa cells from large follicles.

Nonetheless, interesting differences were found between granulosa cells from the two follicle sizes. Although unstimulated aromatase activity was similar for both types, insulin- or FSH and insulin-stimulated aromatase activity was much higher in granulosa cells obtained from the large follicles than from the small ones, showing that aromatase is more sensitive to induction in the former cells (Figure 1B).

**Progestosterone production**

Progestosterone production by granulosa cells obtained from large follicles showed a slight, but not significant decrease compared with basal production after treatment with FSH alone (50 ng/ml) and insulin alone (25 µg/ml) (Figure 2A). This slight decrease appeared to disappear with concurrent treatment with FSH and insulin.

In contrast, granulosa cells from small follicles behaved very differently (Figure 2B). Progestosterone production was not enhanced by adding FSH (50 ng/ml), but it was markedly increased after treatment with a high dose of insulin (25 µg/ml). In these cells, the addition of FSH plus insulin caused an inhibition of insulin action, lowering progesterone concentrations to control values.

Basal progesterone production was significantly higher (~2-fold; \( P < 0.01 \)) in cells from large follicles compared with those from small follicles.

In order to investigate more deeply the steroidogenic activity of granulosa cells from different sized follicles, we also evaluated the aromatase/progesterone ratio (Figure 3). These values were significantly increased after treatment with insulin (25 µg/ml) and FSH plus insulin (FSH 50 ng/ml and insulin 25 µg/ml) in granulosa cells from both large and small follicles.
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Figure 3. Aromatase/progesterone ratio in granulosa cells from large (≥18 mm, A) and small (<15 mm, B). The results are expressed as mean ± SEM of 10 experiments. Values with asterisks differ significantly (**P < 0.01, *P < 0.05) from the control (C). Values with circles are significantly different between the two groups (***P < 0.01). FSH = follicle stimulating hormone; INS = insulin.

Figure 4. Effect of follicle stimulating hormone (FSH) and insulin (INS) on prostaglandin E₂ release by granulosa cells from large (≥18 mm, A) and small (<15 mm, B) follicles. The results are expressed as mean ± SEM of 10 experiments. Values with asterisks differ significantly (**P < 0.05) from the control (C). Values with circles are significantly different between the two groups (°°P < 0.01).

No significant effect was observed with FSH (50 ng/ml) alone compared with basal values. Important differences were highlighted by the aromatase/progesterone ratio between the two cell types: the values were much higher in granulosa cells from large follicles after treatment with insulin (25 µg/ml) or FSH (50 ng/ml) plus insulin (25 µg/ml).

Prostaglandin E₂
Concurrent treatment of granulosa cells with FSH (50 ng/ml) and high doses of insulin (25 µg/ml) increased basal secretion of prostaglandin E₂. However, this effect was significant (P < 0.05) only in granulosa cells harvested from the largest follicles (≥18 mm); in these cells, basal and stimulated prostaglandin E₂ production were almost 3-fold higher than in granulosa cells from small (<15 mm) follicles. These data are shown in Figure 4.

Cyclic AMP
As illustrated in Figure 5, cAMP generation did not show any significant difference between granulosa cells from large and small follicles with regard to basal levels and the values after treatment with FSH (50 ng/ml) plus insulin (25 µg/ml). No differences were observed between the two cell types.

Discussion
In our study, we used granulosa cells from stimulated ovaries previously inhibited by long-term administration of GnRHa. We did not compare different clinical regimens, but rather the response of granulosa cells to general stimuli in different sized follicles from which oocytes at the same stage of maturity had been retrieved.

In stimulated cycles, Polan et al. (1984) found an inverse correlation between aromatase activity and oocyte maturation. Conversely, Garzo and Dorrington (1984) reported that aromatase activity was undetectable in human stimulated follicles with a diameter <1 cm, thereby demonstrating a positive correlation between aromatase activity and diameter of follicles.

Moreover, Lee et al. (1987) have shown that, in stimulated ovaries after treatment with gonadotrophins without GnRHa, low follicular fluid oestradiol concentrations were associated with fertilized but uncleaved eggs. Our results clearly showed that aromatase activity appeared to be more easily elicited after stimulation with FSH and insulin in granulosa cells from the large follicles, thereby indicating no linkage between aromatase activity and apparent oocyte maturity. Granulosa cells from small follicles not only showed lower aromatase activity, but the basal progesterone production was also found to be lower. The greater steroidogenic activity shown by granulosa cells from large follicles could be ascribed to a number of factors acting by paracrine or autocrine modalities.

Table 1 shows the linear correlation between oestradiol peak values and follicles with diameter ≥18 mm and <15 mm. The finding that the number of small follicles (and not the number of large ones) was associated with the oestradiol peak concentration, also reported by other authors (Testart and Amiel, 1991), could be explained by the increased number of small follicles observed in these cycles compared with cycles in which only clomiphene or human menopausal gonadotrophin was used.

Progesterone production in our granulosa cell cultures showed a slight but not significant decrease after treatment.
with FSH alone and insulin alone in granulosa cells from large follicles; to the contrary, insulin alone clearly stimulated progesterone accumulation in granulosa cells from small follicles. These data are intriguing. In many papers (Hurwitz et al., 1987) and also in a recent paper of our own (Andreani et al., 1994), the induction of progesterone production by insulin has been reported in a mixed pool of granulosa cells from different sized follicles (all $\geq$ 16 mm). In this study, we found that insulin-stimulated progesterone production depended on the number of cells from small follicles present in culture. It is particularly surprising that the apparently depressing effect on progesterone production of FSH and insulin independently was abolished by concomitant treatment with the two substances. A number of hypotheses could be envisaged. For example, basal progesterone concentrations in culture may be principally due to granulosa cells from large follicles at the time of retrieval which subsequently become insensitive to further stimuli, while the progesterone production in small follicles may continue to be stimulated by insulin. Another hypothesis could be that insulin or FSH may induce an increment of progesterone metabolism via 20α-hydroxyprogesterone that in granulosa cells from large follicles could be higher than in those from small follicles (Pellicer et al., 1992), and this change could be associated with an increase in aromatase activity (Hsueh et al., 1984). In the light of this, we evaluated the aromatase activity/progesterone ratio and found that the presence of insulin increases this ratio in both types of cells; moreover, cells from large follicles showed higher values compared with those from small follicles. In spontaneous cycles it has been demonstrated that the resumption of oocyte meiosis is followed by a rapid fall in follicular oestradiol due to a reduction in aromatase activity accompanied by a great increase in progesterone production, suggesting that a decrease in oestradiol/progesterone ratio could be a good criterion of follicular maturity (Brailly et al., 1981). In this study, in which stimulated follicles with oocytes at the same stage of maturity were investigated, it would appear that aromatase activity prevails over progesterone production in both types of follicles, even though this finding was more pronounced in granulosa cells from large follicles. A high aromatase/progesterone ratio could also be considered to be a good criterion of follicular maturity. On the other hand, other important intrafollicular markers, such as prostaglandins and purins, should be considered with reference to oocyte quality, as they are correlated with oocyte maturation. Prostaglandins are thought to have a potential modulatory role in oocyte maturation (Jeremy et al., 1987), since a high follicular fluid concentration of prostaglandin $E_2$ has been correlated with oocyte maturity. We found higher amounts of prostaglandin $E_2$ were released from granulosa cells from large follicles compared with those from small follicles (Ekholm et al., 1982), suggesting a direct relationship with growth rather than with oocyte maturity. Several studies support the hypothesis that cAMP is of central importance in the regulation of oocyte maturation. In fact, some substances, such as the vasoactive intestinal peptide, have been demonstrated to stimulate cAMP accumulation from isolated follicles, thereby inducing oocyte maturation (Carlsson et al., 1987). Moreover, Kasson et al. (1985) showed a direct effect of this substance in stimulating cAMP production by rat granulosa cells in vitro. These data are consistent with the hypothesis that an increased production of cAMP by granulosa cells is correlated with oocyte maturation.

In our study, there was no difference in cAMP accumulation between granulosa cells from different sized follicles. This finding could be related to the similar stage of maturity achieved by oocytes harvested from the two types of follicles. Some authors have found that cAMP formation is normally stimulated by gonadotrophins, both in vivo and in vitro (Knecht et al., 1981; Yong et al., 1992). In our study, no stimulatory effect was seen after in-vitro treatment with FSH and insulin. This finding could be ascribed to two different possible mechanisms. First, granulosa cells could be already maximally stimulated by exogenous gonadotrophin therapy, and could then become insensitive to further stimulation in vitro. A second possible explanation could be that the effectiveness of FSH and insulin may be abolished by GnRHa treatment. In this context, Knecht et al. (1981) showed that FSH-induced cAMP production was inhibited by concomitant in-vitro GnRHa treatment.

In conclusion, we found significant biological differences between granulosa cells harvested from large and small follicles containing oocytes apparently at the same stage of maturity. Some of the functional parameters of granulosa cells from large follicles were both more highly expressed at a basal level (progesterone and prostaglandin $E_2$ production) and more inducible (prostaglandin $E_2$ secretion and aromatase activity) than observed in cells from small follicles. In the light of this evidence, follicular size could be considered to be an indirect sign of follicular maturity. Generally, GnRHa plus gonadotrophin treatment leads to a greater number of recruitable follicles, which in turn is associated with a greater proportion of small follicles. However, notwithstanding an apparently comparable oocyte maturity, it can be assumed that the oocytes with a poor ability to develop come mostly from the cohort of small follicles at the time of HCG administration.

The available data regarding the fertilization and implantation rates of oocytes retrieved during GnRHa-treated cycles are conflicting (Nayudu et al., 1989; Tavmergen et al., 1992). However, the observations reported in the present paper seem to support the hypothesis that the lower fertilization and implantation rates observed in GnRHa-treated cycles could be related to the less mature follicular milieu surrounding oocytes in small follicles. Further investigation is required to determine how the follicular environment may affect the maturity of the oocyte and its capacity to develop after fertilization.

References


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