Human ovarian granulosa cells and follicular fluid indices: the relationship to oocyte maturity and fertilization in vitro

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The study investigates the correlation between oocyte maturity and fertilization and a variety of hormonal parameters in follicular fluid and ovarian granulosa cells. A methodology for purification of granulosa cells from contaminating blood cells is also established. A total of 63 follicular aspirates were collected at oocyte retrieval from 30 women superovulated using the long luteinizing hormone-releasing hormone (LHRH analogue)/human menopausal gonadotrophin regimen. Oestradiol, progesterone, testosterone and human chorionic gonadotrophin (HCG) were quantified in follicular fluid and granulosa cells were immunostained for human chorionic gonadotrophin.

Introduction

The widespread use of in-vitro fertilization (IVF)/gamete intra-Fallopian transfer (GIFT) procedures has made ovarian granulosa cells more accessible to research. Granulosa cells from individual follicles may be assessed morphologically, immunohistochemically or biochemically, and these variables can be related to oocyte maturity and fertilization rates.

Aspirates from ovarian follicles contain a heterogeneous cell population representing granulosa cells at different stages of luteal differentiation. The midcycle luteinizing hormone (LH) surge, or human chorionic gonadotrophin (HCG) administration in superovulatory cycles, stimulates granulosa cells to luteinize by binding to cell surface receptors (Whitman et al., 1989). Subsequently, the level of LH/HCG receptors determines the response of these cells to the LH/HCG surge with an increase in progesterone synthesis.

Numerous studies have been conducted on granulosa cells including steroidogenesis in vitro (Bernhisel et al., 1987), ultrastructure and morphometric characterization (Rotmensch et al., 1986) and the study of LH/HCG receptors (Rodriguez et al., 1990).

Internalization of HCG by granulosa cells has been reported in relation to oocyte quality and fertilization (Whitman et al., 1988). However, no characterization of the cells in follicular aspirates was undertaken and hence the quantitative assessment of immunostained granulosa cells was carried out on an undefined population of cells which distorts the statistical results. Purification of granulosa cells from contaminating blood cells is essential prior to immunostaining for HCG.

A possible link has also been suggested between follicular steroids, oocyte maturity and fertilization (Botero-Ruiz et al., 1984; Ellsworth et al., 1984; Brzyski et al., 1990) but no consensus exists between these reports.

The aim of this study was to investigate the correlation between oocyte maturity and fertilization and a variety of hormonal parameters in follicular fluid and ovarian granulosa cells. A methodology for purification of granulosa cells from contaminating blood cells was also established.

Materials and methods

Thirty patients who underwent oocyte recovery at the Reproductive Medicine Unit, Department of Obstetrics and Gynaecology, University Hospital of South Manchester were recruited for this study. Patients’ age ranged from 27 to 41 years (mean 35 years). The causes of infertility included tubal factor (n = 8), endometriosis (n = 9), unexplained infertility (n = 10) and cervical hostility (n = 3). Couples with male factor infertility were excluded from the study. Twenty-one patients had primary infertility whereas nine had secondary infertility.

All women received the luteinizing hormone-releasing hormone analogue (LHRHa, Goserelin; Zoladex; Zeneca, Macclesfield, UK) in the midluteal phase. Down-regulation was confirmed by the onset of menstruation, measurement of oestradiol concentrations, absence of ovarian follicles >9 mm in diameter and an endometrial thickness <8 mm measured by ultrasound scanning (ATL Ultramark 4, 5 MHz probe). Human menopausal gonadotrophin [HMG, Pergonal; 75 IU follicle stimulating hormone (FSH), 75 IU LH; Serono, Welwyn Garden City, UK] two to four ampoules daily were then commenced. Maturation of oocytes was induced by 10 000 IU HCG (Profasi;
Serocyte retrieval was carried out transvaginally 34–36 h later. Follicular aspirates were examined for granulosa cells and oocytes. The latter were graded as follows: grade 4 (cumulus cells tightly packed around the egg), grade 3 (cumulus cells start to expand but still a tight layer of corona cells around the egg), grade 2 (cumulus cells expanded and corona cells still surround the egg but not as tight as in grade 3), grade 1 (cumulus and corona cells well expanded).

Oocytes were inseminated with husbands’ semen at a concentration of 25 000–30 000 spermatozoa per oocyte and examined for pronucleus formation 14–18 h later.

Removal of contaminating blood cells

Cells were isolated from follicular fluid by centrifugation at 900 g for 15 min. The supernatant was removed and the pellet suspended in up to 3 ml Dulbecco’s phosphate-buffered saline (DPBS, pH 7.3; Sigma, Dorset, UK), layered onto 3 ml Histopaque-1077 (Sigma) and centrifuged at 400 g for 30 min. Erythrocytes and polymorphonuclear leukocytes were sedimented; the interface layer of mononuclear cells contained granulosa cells, lymphocytes, monocytes and macrophages. Cells were aspirated, suspended in 10 ml DPBS, pelleted and finally resuspended in 2 ml DPBS.

Cytospins were prepared from each follicular aspirate and analysed cytochemically using May–Grunwald–Giemsa stain (Dacie et al., 1991). These were immunostained with mouse anti-human leukocyte common antigen (LCA) antibody (McAbCD45; Dako, Cambridge, UK) then visualized by indirect immunofluorescence. If the level of leukocyte contamination exceeded 5%, contaminating cells were removed using immunomagnetic beads as follows: Monoclonal anti-leukocyte contamination exceeded 5%, contaminating cells were removed using immunomagnetic beads as follows: Monoclonal antibody to CD45 (273 µl antibody). These were incubated in 68% of the follicular aspirates studied. After this procedure, leukocytes rosetted with immunomagnetic beads were then

Coated particles were stored as a suspension containing 4 x 10^5 beads/ml (30 g/ml) in DPBS/BSA at 4°C. Coated immunomagnetic beads (10 µl) were added to the heterogeneous granulosa cell suspension in each follicular aspirate and incubated for 30 min with rotation. Leukocytes rosetted with immunomagnetic beads were then removed using a Dynal magnetic particle concentrator (Dynal).

Immunocytochemistry of HCG on granulosa cells

Cytospins were prepared from the purified granulosa cell suspensions and stained for HCG using an indirect immunofluorescence technique. These were fixed using 2% formaldehyde in DPBS and incubated at room temperature for 1 h with 30 µl anti-HCG primary rabbit polyclonal antibody (Dako, Cambridge, UK; 1:30 v/v in DPBS). The slides were rinsed in three changes of DPBS (10 min each) and incubated for 1 h with 30 µl (1:20 v/v) of fluorescein-conjugated FITC mouse anti-rabbit IgG (Sigma).

Finally, cytospins were mounted on glass slides using Immumount (Shandon, Runcorn, UK). Negative controls, in which DPBS/BSA (4 mg/ml) was applied instead of primary antibody were included in each experiment. Placental cyrosections (positive controls) were also stained using the same technique. All preparations were examined using a x40 oil immersion objective of a fluorescence microscope (Zeiss, Oberkochen, Germany). Four microscopic fields, containing ~300 cells from each cytospin, were evaluated for immunobound HCG.

Biochemical assays

Follicular fluids were analysed for oestradiol, progesterone, testosterone and HCG using solid phase fluorimmunoassay (Delfia kits; Wallac, Turku, Finland). High concentrations of oestradiol and progesterone in follicular fluid necessitated dilution of follicular samples with hormone-free serum (eestradiol) and male serum (progesterone). The inter-assay coefficients of variation were 5% at a level of 1.8 nmol/l for oestradiol, 6.5% at a level of 21 nmol/l for progesterone, 4% at a level of 44 nmol/l for testosterone and 11.3% at a level of 106 IU/l for HCG. All samples were assayed in duplicate.

Statistical analysis

Data were analysed using multiple regression (analysis of variance) methods. All computations were performed using the GLIM 3.77 computer package. Statistical significance was set at the conventional 5% level. Data are presented as mean with 95% confidence intervals.

Results

Granulosa cells and oocytes were isolated from 63 pre-ovulatory follicles. Oocytes were graded as follows: eight grade 3, 25 grade 2 and 30 grade 1. All oocytes were inseminated for IVF. A total of 38 were fertilized (eight grade 2 and 30 grade 1), 20 did not fertilize (six grade 3 and 14 grade 2) and five showed polyspermic fertilization (two grade 3 and three grade 2).

Table I presents follicular fluid steroids and HCG levels in relation to oocyte maturity. Mean HCG values were significantly high in follicles with grade 3 (immature) oocytes (P = 0.002) whereas oestradiol, progesterone, and testosterone levels did not differ significantly between oocyte maturity grades.

Table I also presents the percentage of granulosa cells immunobound to HCG in relation to oocyte maturity. The percentage of granulosa cells immunobound to HCG varied significantly with the grade of oocyte maturity (P < 0.001), being significantly high in follicles with grade 1 oocytes (13.04%).

Density gradient centrifugation using Histopaque provided a population of granulosa cells with leukocyte contamination ranging from 4 to 15% as revealed by May–Grunwald–Giemsa stain and anti-CD45. Cytospins with leukocyte contamination >5% were subjected to immunopurification. This was required in 68% of the follicular aspirates studied. After this procedure, staining with May–Grunwald stain revealed that leukocytes were reduced to <2% of the total cell count. Table II presents follicular fluid hormone levels in relation to oocyte fertilization. The correlation was not significant.

Table II also presents the percentage of granulosa cells immunobound to HCG in relation to oocyte fertilization. The percentage of granulosa cells immunobound to HCG varied significantly in relation to fertilization (P = 0.020), being significantly high in follicles whose oocytes fertilized normally (10.64%) or showed polyspermic fertilization (9.88%).

Discussion

Granulosa cells are among the most well studied of all endocrine cells because of their easy availability in IVF cycles. Different techniques for the preparation and purification of
granulosa cells have been described which result in different amounts of blood cell contamination (Beckman et al., 1985). Blood cells secrete a variety of hydrolytic enzymes, cytokines and other products that modulate granulosa cell properties in culture (Davis et al., 1979; Halme et al., 1985). In this study, immunopurification yielded a purified granulosa cell population from individual follicles. An accurate quantitation of the percentage of granulosa cells immunobound to HCG was obtained as a fractional proportion of the total population of purified granulosa cells. Bias was eliminated by removal of the non-granulosa cell population. The technique was successfully used prior to HCG immunostaining of granulosa cells (Whitman et al., 1979; Hill et al., 1987). In this study, the immunolocalization of HCG on granulosa cells of pre-ovulatory follicles. Recently, it has been suggested (Aston et al., 1996) that HCG plays a role in granulosa cell adhesion to a glass or plastic-coated extracellular matrix and hence suppresses the release of cells from cultures. The dose of HCG required for the effect on cell retention on granulosa cell adhesion to a glass or plastic-coated extracellular matrix was similar to that required for stimulation of progesterone production.

In this study, a negative selection approach was applied to deplete the mononuclear leukocytes expressing a specific surface marker (CD45) rather than positive selection of granulosa cells. The technique did not affect the functional capacity nor HCG binding to granulosa cells. Positive selection may have strongly reduced the functional capacity of granulosa cells, probably as a result of the physical stress on the cells created by the magnetic beads employed. Moreover, the relatively large magnetic particles (4.5 μm) binding to the granulosa cell surface may have interfered with cell adhesion and cell interaction processes (Lea et al., 1985, 1986).

HCG internalization by granulosa cells has been reported in earlier studies using unpurified populations of granulosa cells (Whitman et al., 1988). Our data suggest that follicles whose oocytes were mature and subsequently fertilized had a high percentage of granulosa cells immunobound to HCG. HCG appears in follicular fluid and on granulosa cells following its i.m. administration. Subsequent to binding at the surface membrane receptors on granulosa cells, the HCG–receptor complex is thought to be internalized within the cytoplasm of those cells that begin to differentiate into luteal cells and synthesize progesterone (Catt et al., 1979; Hill et al., 1987). In this study, the immunolocalization of HCG on granulosa cells taken from individual follicles confirms earlier reports (Rodriguez et al., 1990) on the presence of LH/HCG receptors on granulosa cells of pre-ovulatory follicles. Recently, it has been suggested (Aston et al., 1996) that HCG plays a role in granulosa cell adhesion to a glass or plastic-coated extracellular matrix and hence suppresses the release of cells from cultures. The dose of HCG required for the effect on cell retention on extracellular matrix was similar to that required for stimulation of progesterone production.

In our study, follicles whose oocytes were mature and subsequently fertilized presumably had more granulosa cells with functional LH/HCG receptors and were therefore more responsive to LH/HCG. This provides a more favourable environment for the oocyte as indicated by increased fertilization rate.

HCG concentrations in follicular fluid differed, with the

| Table I. Follicular fluid steroids, human chorionic gonadotrophin (HCG) concentration and the percentage of granulosa cells immunobound to HCG in relation to oocyte maturity (mean with 95% confidence intervals) |
|---|---|---|---|---|---|
| Oocyte maturity (n) | Oestradiol (nmol/l) | Progesterone (nmol/l) | Testosterone (nmol/l) | HCG (U/l) | % of cells immunobound to HCG |
| Grade 3 (8) | 2021 (1359–2980) | 46 767 (29 693–63 840) | 8.31 (5.29–13.05) | 166.2* (87.44–316.1) | 5.39 (4.33–6.70) |
| Grade 2 (25) | 2159 (1721–2708) | 54 797 (44 939–64 654) | 7.05 (5.34–9.14) | 51.33 (35.42–74.38) | 8.4 (7.76–9.19) |

*Mean HCG levels are significantly high in follicles with grade 3 (immature) oocytes (P = 0.002).

| Table II. Follicular fluid steroids, human chorionic gonadotrophin (HCG) concentrations and percentage of granulosa cells immunobound to HCG in relation to oocyte fertilization (mean with 95% confidence intervals) |
|---|---|---|---|---|---|
| Oocyte maturity (n) | Oestradiol (nmol/l) | Progesterone (nmol/l) | Testosterone (nmol/l) | HCG (U/l) | % of cells immunobound to HCG |
| Fertilized (38) | 1830 (1523–2213) | 60 019 (51 590–68 448) | 6.18 (4.94–7.74) | 42.06 (28.13–62.89) | 10.64* (9.50–11.90) |
| Not fertilized (20) | 2119 (1737–2586) | 50 965 (42 161–59 768) | 7.35 (5.81–9.29) | 58.99 (38.76–89.80) | 7.76 |
| Polyspermic fertilization (5) | 2523 (1583–4022) | 50 935 (36 288–77 582) | 7.90 (5.45–13.7) | 83.75 (61.26–98.26) | 9.88* (7.19–13.43) |

*Mean percentage of granulosa cells immunobound to HCG is significantly high in follicles yielding oocytes that subsequently fertilized (P = 0.02).
extent of oocyte maturity being significantly high in follicles with immature oocytes. This may suggest that follicles yielding immature oocytes have not developed their full complement of LH/HCG receptors and hence had fewer receptors available to bind HCG which subsequently accumulated in follicular fluid. Similar results were reported by other investigators (Whitman et al., 1988).

Oestradiol and progesterone concentrations in follicular fluid did not differ significantly in relation to oocyte maturity and fertilization. This confirms the observations of others (Messinis et al., 1987; Stone et al., 1988; Tavmergen et al., 1992; Enien et al., 1995). However, studies using different ovarian stimulation regimens (HMG, FSH and clomiphene citrate/HMG) have suggested that oestradiol and progesterone may be markers for oocyte maturity and fertilization (Fishel et al., 1983; Kreiner et al., 1987).

Concerning testosterone, no correlation was found in relation to oocyte maturity and fertilization. Similar observations were reported (Frederick et al., 1991; Guerrero et al., 1993). However, contradictory results have been obtained by other investigators (Botero-Ruiz et al., 1984; Enien et al., 1995). In fact, the exact role of androgens in follicular development and oocyte maturation is not clear, although it has been suggested that androgens may be necessary for maturation and enhancement of progesterone secretion by granulosa cells (Moon, 1981).

In conclusion, the data suggest that HCG in follicular fluid and on granulosa cells may be relevant to oocyte maturity and the outcome of fertilization. The storage of granulosa cells and follicular fluid at oocyte recovery could therefore allow retrospective analysis should poor or failed fertilization occur. This provides useful information for the evaluation of unsuccessful IVF cycles. Furthermore, immunomagnetic beads provide a reliable procedure for the purification of ovarian granulosa cells from contaminating blood cells.

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