Subpopulations of Human Granulosa–Luteal Cells Obtained from Gonadotropin- or Gonadotropin-Releasing Hormone Agonist/ Gonadotropin-Treated Follicles in In Vitro Fertilization–Embryo Transfer Cycles

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Purpose: Our purpose was to find the differences in granulosa–luteal cells obtained from gonadotropin- versus gonadotropin-releasing hormone (GnRH) agonist/gonadotropin-treated follicles in in vitro fertilization–embryo transfer (IVF-ET) cycles.

Methods: Granulosa–luteal cells were obtained from 45 follicles of women undergoing IVF-ET with gonadotropin releasing hormone (GnRH) agonist and human menopausal gonadotropin (hMG) and from 45 follicles of women with hMG IVF-ET cycles. Subpopulations of granulosa–luteal cells were observed by computerized image analysis in which human chorionic gonadotropin (hCG) was localized using immunoperoxidase staining.

Results: The luteinized granulosa–luteal cells from hMG-treated follicles were larger than those from GnRH agonist/hMG-treated follicles. The hMG-treated follicles contained more hCG-stained cells, particularly those with cytoplasmic hCG localization.

Conclusions: We found differences in morphometric characteristics and hCG localization in granulosa–luteal cells obtained from hMG- versus GnRH agonist/hMG-treated follicles. We presume that the results indicate the influence and importance of luteal-phase support on the clinical pregnancy rate in GnRH agonist/hMG-treated IVF-ET cycles.

KEY WORDS: gonadotropin-releasing hormone agonist; human granulosa–luteal cells; human chorionic gonadotropin localization; in vitro fertilization.

INTRODUCTION

The corpus luteum function is determined by follicular development and maturation. The functional character-

istics are dependent on the hormonal microenvironment during the follicular phase (1,2) and also on the method of superovulation. In human menopausal gonadotropin (hMG)-treated in vitro fertilization–embryo transfer (IVF-ET) cycles, the luteal phase was not disturbed, but after combined gonadotropin releasing hormone (GnRH) agonist/hMG stimulation, without luteal supplementation, an inadequate luteal phase has been reported (3,4). Abnormal luteal function occurs at different levels. Smitz *et al.* (5) have speculated that a luteal defect could be caused by arrest of the pituitary gonadotropin secretion until the late luteal phase or by delayed secretion of human chorionic gonadotropin (hCG) after GnRH agonist/hMG stimulation. A high percentage of endometrial biopsies taken 6–9 days after the ovulatory stimulus is out of phase, the corpus luteum regresses prematurely, and inadequate rescue of the corpus luteum of pregnancy by endogenous hCG is observed (3,6). Studies of the effect of GnRH agonist on human granulosa–luteal cells showed that in vitro progesterone accumulation was impaired compared to that of gonadotropin-treated cells (7).

The process of luteinization is associated with the plasma membrane luteinizing hormone (LH) receptor content on granulosa cells (8). We found differences in hCG localization in human granulosa–luteal cells obtained from natural versus hMG-treated IVF-ET cycles (9,10). The aim of the present study was to evaluate the differences in human granulosa–luteal cells derived from hMG treated versus combined GnRH agonist/hMG-treated IVF-ET cycles.

MATERIALS AND METHODS

Patients and Protocols

Human granulosa–luteal cells were obtained from two groups of 45 infertile women attending an IVF-

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ET program (mean age, 32.7 ± 2.6). In the first group, ovarian stimulation was performed with hMG (2–3 ampoules i.m. per day; Pergonal; Serono, Freiburg in Breisgau, Germany) starting on day 2 of the cycle.

In the second group of 45 women, every patient was treated with the GnRH agonist buserelin (Suprefact; Hoechst, Frankfurt, Germany), 0.6 mg per day starting on the 22nd day of the menstrual cycle until pituitary desensitization was achieved [serum estradiol (E_2), <0.07 nM; absence of follicles >5 mm in diameter in each ovary and a thin endometrium]. Ovarian stimulation was performed with a combination of 0.6 mg GnRH agonist and 2–3 ampoules hMG per day.

The dose of HMG was regulated individually in both groups. Follicular development was monitored daily by transvaginal ultrasonography starting on day 7 of hMG administration (Bruel and Kjaer Medical; transducer type 1849, 7.0 MHz; Gentofte, Denmark) and by determination of the serum E_2 level. The criterion for ovulation induction was a dominant follicular diameter of 17 mm or more in the hMG-stimulated group and 21–23 mm in the GnRH agonist/hMG-treated group.

Ovulation was induced with 5000 IU hCG (Pregnyl; Organon, Oss, The Netherlands) in both groups. Transvaginal follicular aspiration was performed 34–36 hr after hCG administration.

Isolation of Granulosa–Luteal Cells

After oocytes were removed and identified, the visible granulosa luteal cell clumps were collected separately before individual aspirates were centrifuged (200g, 5 min). The volume of supernatant was measured and stored at -20°C until it was assayed for hCG. The sedimented cells and clumps were resuspended in 1.0 ml of Hams F-10 medium (pH 7.35; Seromed; Biochrom KG, Berlin, Germany). Cell viability was assessed by trypan blue staining ($84.7 \pm 6.9\%$). The cells were fixed with 10% buffered formalin for 30 min in both groups. The cell suspension was placed on four or five glass slides, air-dried, and stained with hematoxylin and eosin for morphometric analysis or immunocytochemically for localization of hCG.

Human Chorionic Gonadotropin Localization and Morphometric Analysis

Human chorionic gonadotropin was localized using immunocytochemical staining procedures (peroxidase–antiperoxidase method) on formalin-fixed cells. Polyclonal rabbit anti-human hCG antibodies (A231, No. 019; Dakopatts, Glostrup, Denmark) were used at a

dilution of 1:200 (9,10). Replacing the hCG antiserum with normal rabbit serum assessed nonspecific staining, and the other negative controls included omitting the first antiserum. For positive controls, sections of placenta were used.

The hCG staining was considered positive when brown precipitates were found on the membrane and/or in the cytoplasm (11). A total of 100 cells was determined for each follicle aspirate using computerized image analysis (9,10).

Granulosa–luteal cell subpopulations were identified by hematoxylin and eosin staining, cell area, and cytoplasmic:nuclear ratio, as described by Whitman *et al.* (8). Luteinized granulosa–luteal cells were identified by the presence of an eosinophilic cytoplasm and classified into three subpopulations: small, medium, and large cells (Fig. 1). Small luteal cells were $<75.0 \mu\text{m}^2$ in area, medium cells ranged from 75.0 to $100.0 \mu\text{m}^2$ in area, and large luteal cells were $>100.0 \mu\text{m}^2$ in area. Nonluteinized cells were observed as one group.

RESULTS

The volume of aspirated hMG-treated follicles was 4.0 ± 0.9 ml and the volume of GnRH agonist/hMG-treated follicles was 3.9 ± 0.7 ml.

We identified four granulosa–luteal cell subpopulations in all 90 follicular samples: nonluteinized and small, medium, and large luteinized cells. Nonluteinized cells were all small. Granulosa–luteal cells from aspirates of GnRH agonist/hMG-treated follicles were smaller ($64.91 \pm 9.87 \mu\text{m}^2$) than those from hMG-

Fig. 1. Granulosa–luteal cells stained with hematoxylin and eosin. Small, a small luteinized cell; Medium, a medium luteinized cell; Large, a large luteinized cell. Original magnification, $\times 850$.

Fig. 2. Cell area (S) of granulosa-luteal cells from hMG-treated follicles (unfilled circles) and from GnRH agonist/hMG-treated follicles (filled squares).

treated follicles ($81.02 \pm 11.24 \mu\text{m}^2$) ($P < 0.001$) (Fig. 2).

The relative number of each granulosa-luteal cell subpopulation is shown in Fig. 3. In aspirates of GnRH agonist/hMG-treated follicles, more small luteinized granulosa-luteal cells and fewer medium and large luteinized granulosa-luteal cells were found ($P < 0.05$ and $P < 0.001$).

The relative numbers of each hCG-stained cell type are presented in Fig. 4. The hMG-treated follicles contained more hCG-stained cells than the GnRH agonist/hMG-treated follicles ($P < 0.01$). The GnRH agonist/hMG-treated follicles contained only 0.7% granulosa-luteal cells with cytoplasmatic hCG localization ($P < 0.01$).

Fig. 3. Distribution of human granulosa-luteal cell subpopulations in the follicular aspirates obtained from hMG-treated cycles (open bars) and GnRH agonist/hMG-treated cycles (hatched bars). Nonlut, hematoxylin and eosin-stained nonluteinized cells; Small, Medium, and Large, luteinized cells. * $P < 0.05$; ** $P < 0.001$.

Fig. 4. Distribution of hCG-stained granulosa-luteal cell subpopulations in the follicular aspirates obtained from hMG-treated cycles (open bars) and GnRH agonist/hMG-treated cycles (hatched bars). Mem, membrane hCG localization; Mem/cyto, membrane and cytoplasmic hCG localization; Cyto, cytoplasmic hCG localization. * $P < 0.01$.

DISCUSSION

We found differences in morphometric characteristics and hCG localization between human granulosa-luteal cells obtained from hMG-treated follicles and those obtained from GnRH agonist/hMG-treated follicles in IVF-ET cycles. The average cell area of granulosa-luteal cells was influenced by the two IVF-ET protocols. The granulosa-luteal cells from GnRH agonist/hMG-treated follicles were smaller than those from hMG-treated follicles. This result may reflect a GnRH agonist influence on follicular and granulosa-luteal cell maturation.

Therefore, GnRH or GnRH-like peptides present in the ovary could be involved in local regulation of ovarian function by binding to the GnRH receptors. GnRH receptor mRNA is expressed in granulosa-luteal cells across different functional stages (12) and GnRH receptors are present only in human granulosa cells at a late stage of follicular maturation (13).

It has been demonstrated that GnRH decreases FSH-stimulated steroidogenesis as well as LH/hCG receptors (7). In our study, aspirates of GnRH agonist/hMG-treated follicles contained 12.9% hCG-stained granulosa-luteal cells, compared to 21.4% in hMG-treated cycles (Fig. 4). These findings confirmed the suggestion that cells treated with GnRH agonist are less luteinized 36 hr after hCG administration (7). The relative number of hCG-stained granulosa-luteal cells might explain different steroidogenic characteristics of individual corpus lutea. It could also be involved in the mechanism of luteal-phase deficiency.

However, the GnRH agonist Buserelin caused time- and dose-dependent inhibition of E₂ and progesterone production in primate granulosa cells (14). A decrease in serum E₂ and progesterone concentrations was found, which became significant from day 10 onward after ovulatory hCG injection, compared to the results from stimulation protocols with hMG (5).

The results of our previous clinical study (15) showed that a luteal-phase support regimen significantly influences the clinical pregnancy rate. The luteal-phase support regimen which was normally used in GnRH agonist/hMG-treated cycles significantly lowered the pregnancy rate when it was applied to hMG-stimulated cycles. Our second clinical study (16) confirmed the previous observations. Our results and other reports (4,17,18) show that the clinical effect of hCG luteal supplementation was time dependent.

The relative lack of progesterone could also suggest poor endometrial maturation in the unsupplemented cycles (19). We presume that the lower number of hCG-stained granulosa-luteal cells obtained from GnRH agonist/hMG-treated follicles influences the functional characteristics of the corpus luteum during the early luteal phase, before theca-derived luteal cells in the midluteal phase respond to hCG with additional progesterone production.

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