Differences in gene expression of granulosa cells from women undergoing controlled ovarian hyperstimulation with either recombinant follicle-stimulating hormone or highly purified human menopausal gonadotropin

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Objective: To investigate the differences in the gene expression profile of granulosa cells from preovulatory follicles after controlled ovarian hyperstimulation (COH) with recombinant follicle-stimulating hormone (FSH) or urinary human menopausal gonadotropin (hMG) FSH.
Design: Prospective randomized study.
Setting: University-based facilities for clinical services and research.
Patient(s): Thirty women undergoing treatment with vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI).
Intervention(s): Patients were randomly allocated to receive recombinant FSH or human (hMG) COH. Granulosa cells were collected from follicular fluid after oocyte retrieval, and mRNA were isolated for gene expression analysis.
Main Outcome Measure(s): General gene expression profile.
Result(s): Ninety-six probe sets (85 genes) showed statistically significant differences in expression level in the two groups of granulosa cells. Expression level of luteinizing hormone/human chorionic gonadotropin (LH/hCG) receptor gene and genes involved in biosynthesis of cholesterol and steroids were expressed at lower levels in the hMG-treated cells; inositol 1,4,5-triphosphate-3-kinase-A and S100-calcium-binding-protein-P (anti-apoptosis protein) were expressed at higher levels in hMG than in recombinant FSH.
Conclusion(s): The different hormone compositions of the two drugs used for COH had a statistically significant impact on the gene expression profile of preovulatory granulosa cells. Some of these genes may be important for periovulatory events, which suggests that the preparation used for COH is important for granulosa cell function and may influence the developmental competence of the oocyte and the function of corpus luteum. (Fertil Steril® 2009;91:1820–30. ©2009 by American Society for Reproductive Medicine.)

Key Words: Granulosa, human, recombinant FSH, hMG, LH receptor

A large number of studies have focused on comparing the effect of recombinant follicle-stimulating hormone (FSH) with urine derived human menopausal gonadotropin (hMG)—which contains FSH (several isoforms), luteinizing hormone (LH), and human chorionic gonadotropin (hCG)—on pregnancy rate, endocrine profile, and follicular fluid biochemical profile as well as embryo quality (1–5). The latest meta-analysis has shown statistically significant higher ongoing pregnancy rates with hMG treatment compared with FSH treatment (6), and a large multicenter study comparing hMG and recombinant FSH in more than 600 in vitro fertilization (IVF) cycles found that patients receiving hMG had more cleavage-stage embryos of high quality when compared with the recombinant FSH group (7). During folliculogenesis and oogenesis, the oocyte is surrounded by granulosa cells, and a bidirectional communication between the oocyte and the granulosa cells takes place via gap-junctions and paracrine factors. This communication has a key role in folliculogenesis and is essential for an oocyte to achieve competence to sustain fertilization and embryogenesis (8). Follicle-

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stimulating hormone is a key hormone in governing granulosa cell function. Granulosa cells are sensitive to FSH from the early stages of folliculogenesis, and in the late luteal phase some of these follicles will be recruited to the final growth phase by the intercycle rise in FSH (9).

Little is known about the extremely complex process that generates a developmentally competent oocyte. It has been suggested that the gene expression level of some genes in the granulosa cells can be used as markers for oocyte quality and hence development potential (10). Only one study has been published on the gene expression profile of human granulosa cells without a preceding in vitro culturing period (11) in which the differences in gene expression profile in mural granulosa cells from normal and poor responders were reported. Our study is the first to report on gene expression profiles from in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) patients receiving different FSH preparations for controlled ovarian hyperstimulation (COH). We investigated the differences in the gene expression profile of the mural granulosa cells from preovulatory follicles after COH with recombinant FSH and hMG, respectively.

MATERIALS AND METHODS

Patients and Treatment Cycle

Human granulosa cells were collected at the Fertility Clinic of Hvidovre Hospital, Denmark. Healthy women with regular cycles who had been referred to IVF or ICSI treatment for male factor infertility, tubal disease, unexplained infertility, and/or mild endometriosis participated in the study after providing written consent. The study was approved by the Danish Institutional Review Board (Approval Number: KF 01 268356).

Thirty women aged 21 to 39 years undergoing COH with a standard long agonist protocol treatment were included in the study. Pituitary down-regulation was performed using 0.4 mg daily of the gonadotropin-releasing hormone (GnRH) agonist Nafarelin (Synarela; Delpharm, Brétigny-Sur-Orge, France) for at least 14 days. Upon confirmation of pituitary desensitization (presence of menstrual bleeding, no ovarian cysts, endometrial thickness <5 mm), 30 patients were randomized to receive either recombinant FSH (Gonal-F; Serono, Geneva, Switzerland) or highly purified hMG (Menopur; Ferring Pharmaceuticals, Copenhagen, Denmark) for 8 to 12 days, dosed individually as previously described elsewhere (12). When the leading follicle(s) were >16 mm, hCG (10,000 IE; Pregnyl; Organon, Copenhagen, Denmark) was administered. Patients with less than four preovulatory follicles were excluded from the study.

Aspiration of the oocytes was performed transvaginally by ultrasound guidance 36 hours after hCG administration. Immediately after isolation of the cumulus–oocyte complexes, the aspirates (follicular fluid, mural granulosa cells, erythrocytes, and leucocytes) were pooled in tubes with ethylenediaminetetraacetic acid (EDTA) on ice. Within 30 minutes, purification was initiated.

Purification Method

After ovum pick-up, 1 mL of the aspirate was used for counting leucocytes and erythrocytes (ADVIA; Bayer Healthcare, Tarrytown, New York). The aspirate was centrifuged (10 minutes at $300 \times g$, $+4°C$), and the pellet was suspended in Mg^{2+} and Ca^{2+} free phosphate-buffered saline (PBS). Erythrocytes were removed by erythrocyte lyses buffer (EL-buffer; Qiagen, Hilden, Germany) followed by leucocytes removal with anti-CD45 and anti-CD15 microbeads with magnetic cell sorting (Dynabeads; Dynal Biotech, Oslo, Norway). The cell counts executed by ADVIA were used for estimating the volume of microbeads needed; 25-μL of microbeads solution was added per 2×10^6 leucocytes.

Two smear slides were prepared before erythrocyte lyses and after leukocyte removal, respectively, to ensure that the removal was successful showing a homogenous population of mural granulosa cells. The smear slides were analyzed by Cellavision DM8 (Cellavision AB, Lund, Sweden), which recorded pictures of the slides in a computer.

The mural granulosa cells were stored in RNAlater (Ambion, Austin, Texas) at $-20°C$ until RNA extraction.

RNA Extraction and Processing for Microarray

Following the instructions of RNeasy mini kit (Qiagen), the total RNA was resuspended in 50 μL of RNAse free water. Extracted RNA was stored at $-80°C$ until microarray analysis. The total RNA was checked for clearly visible 18S and 28S RNA bands and a A260/280 ratio of >1.8 (GeneQuant II; Pharmacia Biotech, Uppsala, Sweden) combined with a RNA Integrity Number above 8 as analyzed by Bioanalyzer (Agilent, Santa Clara, CA) and NanoDrop (NanoDrop Technologies, Wilmington, DE). Samples that met these criteria were labeled for microarray hybridization.

Microarray Analysis

Total RNA extracted from the mural granulosa cells obtained from five patients (0.40 μg of RNA from each) was pooled in groups to give a total of six pooled samples representing two treatment groups (recombinant FSH, Gonal-F; and hMG, Menopur) with three biological replicates in each. The RNA from each pooled sample was labeled according to the Affymetrix (Santa Clara, CA) standard protocol for One-Cycle cDNA synthesis. In short, total RNA is reverse transcribed into double-stranded cDNA using an oligo-d(T)primer containing a T7 RNA polymerase promoter. The cDNA is then in vitro transcribed into labeled cRNA with biotinylated nucleotides. The cRNA is fragmented and hybridized to the Affymetrix GeneChip, then washed and stained with a phycoerythrin-streptavidin conjugate. The labeled mural granulosa samples were hybridized to the Human Genome U133 2.0 Plus array (Affymetrix) that queries the expression of 48,000 well-substantiate genes. Each transcript is represented and queried by one or more probe sets. A probe set comprises 11 probe pairs; each probe pair contains a perfect match and mismatch 25-mer