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Type of gonadotropin during controlled ovarian stimulation affects the endocrine profile in follicular fluid and apoptosis rate in cumulus cells

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ABSTRACT

Objective: To determine whether the type of gonadotropin affects the secretion of oocyte-specific factors, the endocrine pattern in follicular fluid, and the apoptosis rate in cumulus cells.
Study design: Prospective and observational study into an university-affiliated private in vitro fertilization setting. Ninety women included in our oocyte donation program were stimulated with human menopausal gonadotropin (hMG), recombinant follicle-stimulating hormone (FSH) or urinary FSH. Main outcome measures were growth-differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15) expression, hormonal profile and apoptosis rate.
Results: No statistically significant differences were observed for GDF-9 and BMP-15 among the three treatment groups. Estradiol concentrations in follicular fluid were significantly higher in women treated with hMG compared with recombinant FSH or urinary FSH. Testosterone levels were also higher in the group treated with hMG. A statistically significant association was found between the degree of apoptosis in cumulus cells and the type of gonadotropin.
Conclusions: The type of gonadotropin used during controlled ovarian stimulation significantly affects endocrine profiles in follicular fluid and the apoptosis rate in cumulus cells. However, there were no significant differences in the levels of oocyte-secreted factors between treatments.

Introduction

While the contribution of granulosa cells to oocyte development has been studied for many years, it was recently determined that the oocyte itself plays a key role in directing its own fate, as well as the growth and differentiation of the follicle [1]. This regulatory capacity is achieved through the synthesis and secretion of oocyte-specific factors (OSFs), such as growth-differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15), which act on granulose cells to modify their proliferation, function and differentiation [2].

The follicular environment is primarily influenced by the type of gonadotropin the follicle is exposed to during the follicular phase. The role of gonadotropins has been especially important in improving the efficiency of in vitro fertilization. Several studies comparing the use of human menopausal gonadotropin (hMG) with recombinant follicle-stimulating hormone (rFSH) have found significant differences in the endocrinological profile and the follicular dynamics [3–5]. These differences have been related to the human chorionic gonadotropin (hCG)-driven luteinizing hormone (LH) activity added to hMG. Moreover, differences in the proportion of acid residues in FSH molecules should be considered. On the other hand, the main physiological regulatory hormones of follicular survival are the gonadotropins. Suppression of serum gonadotropins leads to massive apoptosis of granulosa cells in developing follicles resulting in atresia [6]; whereas, gonadotropin treatment of early antral and pre-ovulatory follicles prevents this unplanned apoptosis [7].

A key challenge facing reproductive biologists is the integration of this knowledge about oocyte-secreted factors into coherent physiological mechanisms of how oocytes govern folliculogenesis, cumulus cell (CC) function, and oocyte and embryo development. Although key OSFs have been identified, understanding their

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modes of action is complicated by multiple interactions between maternal and oocyte signaling molecules, as well as the constantly changing state of physical interactions between the oocyte and its companion somatic cells during folliculogenesis. Thus, our study aimed to determine if there is any relationship between different gonadotropin preparations and OSFs secretion, the endocrine pattern in follicular fluid, and the apoptosis rate in cumulus cells during controlled ovarian stimulation.

Material and methods

Patient population and ovarian stimulation

This study was approved by an Institutional Review Board (MAD-GV-04-2009-01). It complied with the Spanish law governing Assisted Reproductive Technologies (14/2006). Ninety women included in our oocyte donation program, who were undergoing controlled ovarian stimulation, were enrolled in the study. This size sample was shown to be biologically representative for this pilot study.

Oocyte donors were healthy women aged between 18 and 35 years old, with regular menstrual cycles, no hereditary or chromosomal diseases, normal karyotype and were negative when screened for sexually transmitted diseases [8]. Inclusion in the oocyte donor pool also required that the donor had at least seven antral follicles per ovary at the beginning of the cycle. Donors who had polycystic ovary syndrome, based on Rotterdam criteria [9] or multifollicular ovaries, were excluded.

Patient assignment to each group was made based on a quasi-experimental design comprising consecutive opportunity sampling. Treatment groups were assigned during the control visit at the start of menses, before starting ovarian stimulation. An oral contraceptive pill (Microgynon30®, Bayer Hispania, Spain) was taken for a maximum of 21 days, starting on day 1–2 of the menses of the previous cycle. After a wash-period of five days after the last pill, donors started with their assigned stimulation protocol. Subjects were allocated to receive daily doses of 150–300 UI of rFSH (Gonal-F®, Merck-Serono, Spain; $n = 30$), urinary FSH (uFSH) (Fostipur®, Angelini, Spain; $n = 30$) or hMG (HMG-Lepori®, Angelini, Spain; $n = 30$) depending on their age, body mass index (BMI) and ovarian response in previous cycles. Daily doses of 0.25 mg gonadotropin- releasing hormone antagonist cetrorelix (Cetrotide®, Merck-Serono, Spain) were started on day six of stimulation in each group. When at least three or more leading follicles reached a mean diameter of $\geq$18 mm, hCG (Ovitrelle®, 250 μg; Merck-Serono, Spain) was administered subcutaneously, and transvaginal oocyte retrieval was performed 36 h later.

Cumulus cell collection

Immediately after oocyte retrieval, cumulus-oocyte complex (COC) were collected and incubated in individually droplets in equilibrated (6.5% CO_2, 37 °C) fertilization medium (Global for Fertilization, Global, Canada) for 3 h prior to cumulus removal. Shortly before ICSI, individual COC were dissociated. Cumulus cells were separated mechanically from the oocyte with strippers after brief exposure to hyaluronidase (Vitrolife, Sweden). Cumulus samples were then collected into individual microcentrifuge tubes, briefly centrifuged, and the supernatant removed to leave a pellet of cumulus cells. About 100 μl lysis RLT buffer (Qiagen) was added to each sample, which was immediately transferred to ice and then stored at −80 °C until analysis.

The maturity of denuded oocytes was immediately assessed following removal of cumulus cells. Individual oocytes were categorized as germinal vesicle (GV), metaphase I (MI) or metaphase II (MII).

Determination of GDF-9 and BMP-15 expression levels in follicular fluid

Levels of GDF-9 in follicular fluid were estimated by a sandwich enzyme-linked immunosorbent assay (ELISA) using the commercial kit Quantikine® (R&D Systems, MN, USA), according to the instructions of the manufacturer. A calibration curve was constructed by plotting absorbance values against concentrations of calibrators. Absorbance at 450 nm was read and concentrations of each sample were determined using the calibration curve. The minimum detectable dose (MMD) of GDF-9 ranged from 0.0 to 4.4 pg/ml.

The concentration of BMP-15 was determined by Western blot. Fifteen μg of protein from follicular fluid was loaded onto a 10% sodium dodecyl sulfate gel and electrophoresed under non-reducing conditions. The separated proteins were transferred onto nitrocellulose membranes by applying 290 mA for 1 h. The blots were blocked for 1 h at room temperature in phosphate-buffered saline (PBS) containing 5% non-fat dry milk. Subsequently, the blots were incubated for 1 h with an antibody against BMP-15 (1:500; Santa Cruz Biotechnology sc-28911) overnight at 4 °C. After washing three times with PBS, the blots were incubated with rabbit antigoat secondary antibodies (1:2000; HRP, sc-2768) during 1 h at room temperature. Then, the blots were washed again with PBS. The HRP-bound secondary antibody was detected with the LAS3000 Fujifilm device. The relative densities of the bands were expressed as arbitrary absorbance units per area; β-actin was used as internal control. All samples were electrophoresed and analyzed in duplicate and values were averaged before statistical analysis.

Hormonal determinations in follicular fluid

The levels of estradiol (E2), testosterone (T), progesterone (P4) and FSH in follicular fluid were all determined by chemiluminiscence using the Architect analyzer (Abbot Diagnostic, Spain).

Detection of apoptosis in cumulus cells

Apoptotic cells were quantified on the basis of externalization of phosphatidylserine (PS) to the outer layer of plasma membrane during apoptosis, according to the manufacturer's instructions for annexin V-FITC (Miltenyi Biotech, Auburn, USA). Data analysis was performed with the Cell Quest software (Becton-Dickinson, NJ, USA). Ten thousand cells were analyzed in each treatment.

For the study of DNA fragmentation by the terminal desoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method, we used the In Situ Cell Detection kit (Roche Diagnostics, Manheim, Germany) according to the manufacturer's instructions. The DNA fragmentation in cumulus cells was assessed using the In Situ Cell Detection kit (Roche Diagnostics, Manheim, Germany). Fluorescence was measured in the same device and with the same conditions we mentioned above (FACScan, Becton-Dickinson, NJ, USA). A negative control was obtained by omitting the terminal desoxynucleotidyl transferase (TdT) enzyme in the reaction mixture during the labeling step.

Statistical analysis

The levels of oocyte-secreted factors and steroids in follicular fluid, as well as the percentage of apoptotic cells were expressed as the means ± standard deviation (SD). The data for the apoptotic cells obtained after annexin V and TUNEL binding analysis underwent arcsine transformation to obtain a normal distribution. The differences between treatment groups were assessed by two-way ANOVA analysis for continuous variables and the Chi-squared test for percentages, with significance set at $p < 0.05$.