Lower apoptosis rate in human cumulus cells after administration of recombinant luteinizing hormone to women undergoing ovarian stimulation for in vitro fertilization procedures

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Objective: To investigate the effects of recombinant (r-) LH supplementation in "low responder" patients undergoing ovarian stimulation with r-FSH for an IVF program. The apoptosis rate in cumulus cells was used as an indicator of oocyte quality.

Design: Comparison of the rate of DNA fragmentation and caspase-3 activity in cumulus cells in women stimulated with r-LH and r-FSH, versus patients treated with r-FSH alone (control).

Setting: In vitro fertilization (IVF) laboratory.

Patient(s): Forty patients undergoing assisted fertilization programs treated with a GnRH agonist, or r-FSH treatment begun on day 3 of the cycle (control). In the r-LH group, from day 8 of gonadotropin stimulation, 150 IU per day of r-LH were administered.

Intervention(s): Terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine-triphosphate (dUTP) nick-end labeling (TUNEL) assay, and anti-caspase-3 cleaved immunoassay, to detect apoptosis in human cumulus cells.

Main Outcome Measure(s): Difference in DNA fragmentation rate between cumulus cells derived from r-LH treatment and cumulus cells derived from control patients.

Result(s): No differences were observed between the two groups in the total amount of r-FSH administered and in the number of retrieved oocytes per patient. A statistically significant increase in the number of immature oocytes and in the E_2 serum peak was observed in the control group. The number of transferred embryos was significantly higher in the r-LH group. Pregnancy and implantation rates were higher in the r-LH group, but without statistical significance. The apoptosis rate in cumulus cells was higher in the control group than in the r-LH group.

Conclusion(s): This study suggests that supplementation with r-LH improves the chromatin quality of cumulus cells involved in the control of oocyte maturation. (Fertil Steril® 2007;87:542–6. ©2007 by American Society for Reproductive Medicine.)

Key Words: Human cumulus cells, apoptosis, IVF, pregnancy rate, implantation rate, recombinant LH, oocyte quality

Normally, functional ovarian activity involves the synergic action of the two pituitary gonadotropins, FSH and LH, which determine follicular growth and maturation, ovulation, and luteinization. Their combined action on granulosa cells, and on the internal theca, is expressed in the "two cells-two gonadotrophins" theory (1). Years of experience in inducing multiple follicular growth has shown that ovarian follicular growth proceeds normally in the presence of very small quantities of endogenous LH. In other words, endogenous LH is able, in the presence of pharmacological doses of FSH, to allow adequate follicular steroidogenesis, even when present in very low serum concentrations, as it need be tied only to <1% of its receptors expressed by the theca cells (2).

Our interest was in investigating normogonadotropic women who, when undergoing induction of multiple follicular growth, showed an insufficient ovarian response in terms of follicular growth (defined as "low responders"), despite an increase in the units of gonadotropin administered. Hence, the administration of GnRH could completely suppress the secretion of endogenous LH, without any exogenous support to maintain the biological activity of the LH. In this study, we tried to verify the efficiency of supplementation with exogenous LH, during ovarian stimulation, in those patients who had previously experienced failed attempts at assisted fertilization. The clinical effect was assessed in terms of ovarian response, and especially the

influence on the quality of oocytes that were collected. We tried to identify indicators that were not the classic morphological assessment criteria for oocyte quality, but sought rather to establish better oocyte quality through chromatin analysis of the oophorous cumulus cells, which play a vital role in the maturation of oocytes during folliculogenesis. Granulosa cells, as is well-known (3), are highly sensitive to apoptotic pathways, thus most follicles that are selected during the reproductive life of a woman are discarded by atresia, an apoptotic process. The somatic cells of follicles that reach ovulation are supported by specific metabolic pathways that guarantee survival. Such pathways are activated by gonadotropins and by an as yet undefined number of growth factors, which prevent an apoptotic outcome (4). In the patients involved in this study, we verified whether the administration of LH during ovarian stimulation may have conditioned the chromatin pattern of the oophorous cumulus cells, considering this an important marker in defining adequate biological activity that would allow optimum synchronization of cytoplasmic and nuclear maturation of the oocyte.

MATERIALS AND METHODS

A prospective and randomized study was performed with 60 patients (who had undergone previous stimulation of r-FSH >3,000 IU), from September 2004 to February 2005. On day 8 of stimulation, 42 patients were enrolled (Fig. 1), 24 of whom were treated with r-FSH combined with r-LH, while 18 were stimulated with r-FSH and used as a control group. Randomization was realized in blocks of three, using computer-generated random number tables. The sample size of 30 patients in each treatment group was calculated to have 80% power to detect a mean difference of 2.0, with a significance level of 0.01. All patients who presented a normal basic level of FSH (<12 IU/mL) and normal body mass index (BMI = kg/m^2 <28) were treated with a GnRH agonist (buserelin, 0.2 mL/d), starting on day 21 of the previous cycle. The average age was 33.00 years in the LH group, and 36.33 years in the control group. Administration

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FIGURE 1

Flow chart of studied patients.

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of r-LH (75–150 IU/d, Luveris; Serono Pharma, Rome, Italy) in the specific LH group began from day 8 after starting r-FSH stimulation. The stimulation therapy continued with a fixed daily dose of 225 IU of r-FSH (Gonal-F; Serono Pharma, Rome, Italy). Follicular growth was monitored daily using ultrasound and serum Estradiol E$_2$ levels starting on day 6 of stimulation, modifying the dose of r-FSH as a consequence. When, on day 8, patients presented a value of serous E$_2$ <180 pg/mL, and the ovarian condition showed at least six follicles with diameters ranging between 7–10 mm and no follicle with a diameter >12 mm, group randomization was carried out in the two study groups. The ovulatory dose of 10,000 IU of hCG (Gonasi; AMSA SRL, Rome, Italy) was administered when at least 3 or 4 follicles presented a diameter of ≥18 mm. Apoptosis in cumulus cells was examined with the use of a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay and the immunotest for caspase-3.

The pool of cumulus cells was collected after incubating the cumulus-oocyte complex in a solution of hyaluronidase (80 IU/mL, Medicult, Jyllinge, Denmark). The released cells were collected in a test tube (Falcon, Franklin Lakes, NJ) containing 2 mL of basic culture medium (Quinn's advantage medium with HEPES; SAGE IVF, Trumbull, CT) and centrifuged twice at 300 × g for 10 minutes. Oocytes were transferred to a culture medium (fertilization medium, SAGE IVF) and incubated at 37°C, 5% CO$_2$, until the moment of carrying out intracytoplasmic sperm injection (ICSI).

Cumulus cells were then fixed in 3.7% paraformaldehyde for 60 minutes. After centrifuging at 300 × g for 5 minutes, the supernatant was removed, and phosphate-buffered saline (PBS)-glycine was added (0.1 M glycine in PBS and 0.3 mg/mL bovine serum albumin [BSA]). Then the cells were mounted on polylysine-coated glass slides by cytocentrifugation.

Fluorescent TUNEL Assay, or TdT In Situ

Cumulus cells were washed for 5 minutes in 1× PBS (137 mM NaCl, 2.68 mM KC1, 1.47 mM KH$_2$PO$_4$, and 8.1 mM Na$_2$HPO$_4$) and permeated for 10 minutes on ice in 0.1% Triton X100 and 0.1% sodium-citrate in 1× PBS, and then washed three times in 1× PBS at room temperature for 5 minutes each time. Cumulus cells were then incubated for 60 minutes at 37°C in a humidified chamber in 50 μL of a mixture containing 5 μL of nucleotide Mix, 1 μL of TdT enzyme, and 45 μL of equilibration buffer (DeadEnd Fluorometric TUNEL System, Promega Italia SRL, Milano, Italy).

Cumulus cells were incubated with the same mixture without the TdT enzyme (negative control), and another slide was pretreated for 5 minutes with 100 μL of DNAse buffer, and then treated for 10 minutes with a DNAse buffer solution containing 10 unit/mL of DNAse I (positive control).
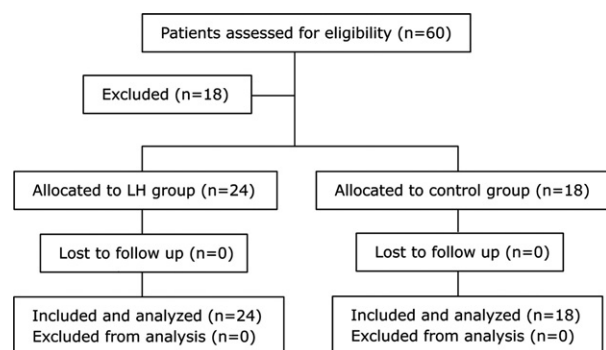
TABLE 1

Results of the trial between patients treated with r-LH and r-FSH (LH group) and patients treated only with r-FSH (control group).

Variables	Control group	LH group	P
Cycles (n)	18	24	
Age (average $\pm$ SD)	36.33 ± 2.1	33.00 ± 3.1	
FSH administered (IU)	$3,644.4 \pm 1,110$	$3,731.2 \pm 1,773$	
E_2 on day of hCG (pg/mL)	$2,640.22 \pm 1,221$	$1,228.00 \pm 830.59$	<.01
Recovered oocytes (n)	7.00 ± 3	5.33 ± 4.8	
GV-MI oocytes (n)	2.33 ± 2	0.58 ± 1.08	<.01
Fertilization rate (%)	85.7	74.6	
Embryos, grades 1–2 (n)	2.11 ± 1.6	2.82 ± 0.4	
Transferred embryos (n)	1.56 ± 1	2.91 ± 0.5	<.01
Pregnancy rate (%)	25.0	45.4	<.01
Implantation rate (%)	12.5	15.6	<.01

Note: GV-MI, Germinal Vesicle-Metaphase I

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Reaction was blocked with 2× SSC for 15 minutes. Three washes of 5 minutes each were carried out in 1× PBS. Cumulus cells were stained with propidium iodide (1 μg/mL) for 10 minutes at room temperature, and observed under a fluorescent microscope equipped with a ×20 0.40 objective.

Anti-Caspase-3 Cleaved Immunoassay

After three washes in 1× PBS (137 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, and 8.1 mM NaH_2PO_4), cumulus cells were permeated for 10 minutes on ice in 0.1% Triton X100 and 0.1% sodium-citrate in 1× PBS. Then after another three rinses with 1× PBS, cells were incubated overnight at 4°C with anti-caspase-3 cleaved polyclonal antibody (Cell Signaling Technology, Inc., Danvers, MA) (1:50 dilution) in 1× PBS containing 3% BSA. In negative controls, the primary antibody was omitted. After three rinses with PBS, anti-rabbit Ig fluorescein-linked (Amersham Biosciences, Milano, Italy) (1:60 dilution in 1× PBS containing 1% BSA) secondary antibody was added to the samples for 60 minutes. Then the secondary antibody surplus was removed by washing three times with PBS. Cumulus cells were stained with propidium iodide (1 μg/mL) for 10 minutes. Slides were mounted in 10 μL DABCO solution (deionized H_2O, 1 M Tris-HCl, pH 8, 2 mM DABCO, and glycerol) for maintaining fluorescence, and were observed under a confocal laser scanning microscope (Olympus FV300, Olympus Italia S.r.l. Segrate (MI)), equipped with green helium-neon (543-nm) and blue argon-ion (488-nm) lasers. Optical sections were captured using an PlanApo ×60 1.40 oil objective.

Statistics

The data obtained in the treatment and control groups were compared using Student's *t*-test and the chi-square test. All reported values are given as 95% confidence intervals. $P<.01$ was considered statistically significant.

RESULTS

In this study, we used the procedures of our previous research to investigate apoptosis in human oocytes (5). A TUNEL assay and immunological tests, performed in situ with the anti-caspase-3 cleaved antibody, allowed us to highlight fragments of DNA and to confirm apoptosis, respectively.

The results showed (Table 1) that there was no statistical difference between the two groups in terms of the quantity of r-FSH administered and the number of collected mature oocytes. A significant statistical difference, however, was recorded in the number of immature oocytes collected (2.33

TABLE 2

TdT assay and caspase-3 immunoassay in cumulus cells.

Study group	Cells (n)	Positive TdT (%)	Positive caspase-3 immunoassay (%)
Control group $\pm$ SD	568 ± 72	18.2	17.0
LH group $\pm$ SD	612 ± 76.5	12.1	11.0

Note: TdT, terminal deoxynucleotidyl transferase

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in the control group, versus 0.58 in the LH group; $P<.01$), in the quantity of estrogen at the time of induction of ovulation (2,640.22 Pgr/mL in the control group, versus 1,228.0 Pgr/mL in LH group; $P<.01$), and in the number of embryos transferred, with the highest result in the LH group (2.91 versus 1.56; $P<.01$).

A significant difference was found in the LH group, where a higher pregnancy rate (PR) (45.4% vs. 25%) and implantation rate (15.6% versus 12.5%) have been recorded in the LH group. The study of apoptosis in cumulus cells (Table 2) showed a higher rate of cells with chromatin fragmentation in the control group than in the LH group (TdT, 18.2% versus 12.1%). The higher presence of caspase-3 cleaved in the control group than in the LH group (17.0% versus 11.0%) could be a sign of irreversible apoptosis. A similar rate of caspase-3 cleaved and DNA fragmentation was found in both groups.

DISCUSSION

This study focused on verifying whether the administration of LH could increase PR and implantation rate by improving the quality of oocytes collected after multiple follicular growth therapy.

The fundamental role of somatic cells in sustaining adequate ovarian development has been recognized for many years. In 1935 (6), a close connection between the two types of cells, granulosa cells and oocyte, was shown. It was also demonstrated that oocytes collected from preantral follicles spontaneously resume meiosis (gonadotropin-independent) in culture, leading to the conclusion that somatic cells of the cumulus-oocyte complex (COC) keep the oocytes in a state of meiotic arrest. Subsequent studies showed that somatic follicular cells promote meiotic resumption and its continuation into metaphase II (nuclear maturation). Cumulus cells also promote the oocyte "ability" necessary for adequate fertilization and the consecutive embryogenesis that ends with implantation (cytoplasmic maturation). Thus the main role of follicular somatic cells, especially during the final phases of folliculogenesis, consists of synchronizing nuclear maturation with cytoplasmic maturation through the complete suppression of oocyte transcription until nuclear maturity (7–9). Various investigators showed that close molecular communication between oocytes and granulosa cells exists during folliculogenesis (hypothesis of an oocyte-granulosa cell regulatory loop), essential for inducing and coordinating differentiation in the oocyte and in the somatic compartment.

Several molecules are involved in this specific pathway: oocyte growth differentiation factor 9 (10,11) seems to control the physiological syncronization of cytoplasmic and nuclear maturation (12,13). The mRNA of epidermal growth factor (EGF) was also found in granulosa, cumulus, and oocyte cells, and it acts on granulosa and cumulus cells with an antiapoptotic effect (14). Growth differentiation factor-9 synthesis is activated by EGF in the oocyte, with a positive effect on cumulus cells. The expression of EGF seems to be induced by LH in the theca cells, and as a molecular cascade it involves granulosa, cumulus, and oocyte synthesis of EGF (15), preserving these cells from apoptotic destiny.

Our results seem to confirm this scenario. The data suggest that supplementation with r-LH during the late follicular phase improves some clinical parameters. In particular, it seems to reduce significantly the number of immature oocytes collected after pick-up, while it increases the number of embryos transferred, correlated with a lower risk of hyperstimulation, as shown in the lower value of the E_2 plasmatic peak. Furthermore, the increase in PR and implantation rate may be correlated with the reduction of apoptosis seen in the cumulus cells of patients treated with r-LH. This may represent a situation in which, although in the presence of a low quantity of this hormone, because of its direct action on the cumulus and granulosa cells, or because of the paracrine effect mediated by secreting factors in the theca and oocyte cells, the cumulus cells are preserved from apoptosis. Maintaining their physiological function for a longer time, cumulus cells are better able to support nuclear and cytoplasmatic maturation of the oocyte until ovulation, thus allowing the collection of oocytes with better "intrinsic" qualities that are necessary for sustaining fertilization and the early phases of embryogenesis. Hence, if cumulus cells are preserved from apoptotic processes, the oocyte of the COC receives no molecular signal able to activate apoptotic pathways.

Bosco et al. (5) demonstrated that apoptosis in human oocytes determines fertilization failure after ICSI. If the activation of apoptosis in the oocyte is regulated by molecular signals coming from cumulus cells through gap-junctions, we consider a lower apoptotic rate in cumulus cells to be an indicator of good oocyte quality, in terms of a greater capacity to be fertilized and to produce embryos with a higher implantation potential. Further studies will be necessary, especially in the area of molecular communication between the various components of the follicular unit, to provide further support for these data.

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