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Ultrastructural analysis of granulosa cells of IVF patients

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Summary

Objective: To compare the percentage and ultrastructure of normal and abnormal granulosa cells and their effect on fertilization and pregnancy rate between gonadotropin releasing hormone (GnRH) agonist and antagonist treatment. *Materials and Methods:* In this study, granulosa cells obtained from 22 women undergoing in vitro fertilization (IVF) treatment due to unexplained infertility with either with GnRH agonist (n=11) or GnRH antagonist (n=11) were evaluated by light and electron microscopy. *Results:* GnRH agonist and antagonist therapy was found to have no effect in terms of abnormal granulosa cell percentage (0.0679 ± 0.08977 *vs* 0.0481 ± 0.05164; $p > 0.05$), fertilization [85 (45-90) *vs* 75 (64-93)] and pregnancy rate (37% *vs* 46%). Light microscopic observations showed similar features of normal cells of agonist and antagonist-treated cells. Ultrastructural evaluation also revealed that there was no difference between cells of two treatment groups. *Conclusion:* Both GnRH agonist and antagonist treatment for ovarian stimulation may have similar effects on granulosa cells at the morphological and ultrastructural level, as well as on fertilization and pregnancy rates.

Key words: Granulosa cell; Ultrastructure; In vitro fertilization; Fertilization rate; Pregnancy rate.

Introduction

Controlled ovarian hyperstimulation (COH) protocols are widely used to obtain multiple oocytes in vitro fertilization (IVF) programmes. Suppression of gonadotropin secretion can be achieved with either gonadotropin releasing hormone (GnRH) agonists or antagonists. Both groups of drugs are routinely used for ovarian stimulation to prevent luteinizing hormone (LH) surge. However, the use of GnRH antagonists has been shown to result in lower follicular fluid and serum estradiol concentration than GnRH agonist treatment. It has also been known that application of GnRH antagonist protocol decreases the duration of ovarian stimulation and the incidence of ovarian hyperstimulation syndrome [1-3].

In this study, the authors aimed to compare ultrastructural differences and percentage of normal and abnormal isolated human granolusa cells from follicular aspirates of women undergoing IVF treatment with agonist or antagonist protocol.

Materials and Methods

Patients

Granulosa cells were obtained from 22 women undergoing assisted reproduction due to unexplained infertility and treated either with GnRH agonist (n=11) or the GnRH antagonist (n=11). Patient characteristics are shown in Table I.

Stimulation protocols

The 11 patients underwent controlled ovarian hyperstimulation consisting of luteal long leuprolide acetate and recombinant follicle stimulating hormone (FSH) using the step-down protocol. When desensitization was achieved, as evidenced by plasma E2 levels of ≤ 50 pg/ml, the absence of ovarian follicles and endometrial thickness ≤ six mm on transvaginal ultrasound examination [4], daily s.c. injection of recombinant FSH was commenced. The starting dose of gonadotropin was determined based on the age of the female, antral follicle count at baseline transvaginal ultrasonography, day 3 FSH and E2 levels, body mass index (BMI), and previous ovarian response, if available.

The 11 patients in the GnRH antagonist group underwent COH consisting of cetrorelix and recombinant FSH, using the step-down protocol. When desensitization was achieved, as evidenced by plasma E2 levels of 50 pg/ml or less, the absence of ovarian follicles and endometrial thickness six mm or less on transvaginal ultrasound examination, [5] a daily s.c. injection of recombinant FSH (Gonal-F) was commenced. The starting dose of gonadotropin was determined based on female age, antral follicle count at baseline transvaginal ultrasonography, day-3 FSH and estradiol (E2) levels, BMI, and previous ovarian response, if available. Flexible GnRH antagonist protocol was used. If serum E2 level was more than 600 pg/ml and/or if leading follicle exceeding 14 mm in diameter were present, cetrorelix 0.25 mg was initiated as daily injections up to the day of oocyte pick-up. Ovarian response was monitored with frequent serum E2 measurements and transvaginal ultrasonography, as described previously [6]

The criterion for human chorionic gonadotropin (hCG) administration was the presence of three or more follicles exceeding 17 mm in diameter. Oocyte retrieval was carried out under local anesthesia using vaginal ultrasound-guided puncture of follicles 36 hours after hCG administration.

Standard procedures were carried out for gamete-embryo handling and cleavage-stage ET, or blastocyst transfer was performed under abdominal ultrasonography guidance in all cases using a soft catheter. The luteal phase was supported by daily vaginal progesterone suppositories starting at one day after oocyte pick-up. Clin-

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Table 1. — *Patient and cycle characteristics, fertilization rates, embryo data, and clinical outcomes of the two study groups.*

Parameter	GnRH agonist (n=11)	GnRH antagonist (n=11)	*p*-value
Age (years)	31 (29-34)	32 (30-38)	NS
Body mass index (kg/m^2)	28 (21-31)	27 (24-33)	NS
Day of hCG injection	9,00 (8,50-10,50)	10,00 (9,00-11,00)	NS
Total dose of rFSH administered (IU)	2662,50 (2268,75-4875,00)	3000,00 (2325,00-3375,00)	NS
Number of oocytes retrieved	10,00 (4,75-18,25)	14,00 (11,00-23,00)	NS
Number of metaphase II oocytes	7,00 (2,50-12,00)	14,00 (6,00-20,00)	NS
Number of embryos transferred	3,00 (3,00-3,00)	3,00 (3,00-3,00)	NS
Fertilization rate	85 (45-90)	75 (64-93)	NS
Pregnancy rate[a]	%37	%46	NS

[a] Defined as the intrauterine presence of fetal heartbeat as assessed by ultrasound scan. NS = not statistically significant.

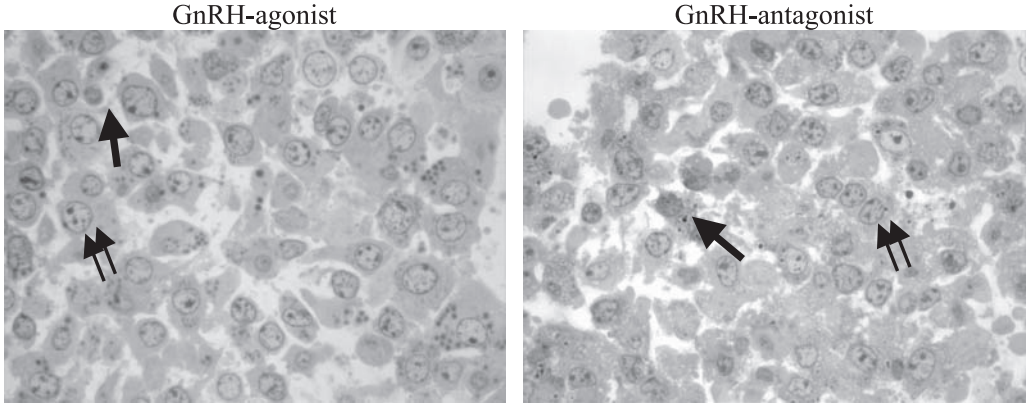

Figure 1. — Light microscopic identification of granulosa cells from GnRH agonist- or antagonist-treated women on semithin sections. Normal (double arrow) and abnormal cells (arrow). Toluidine blue, x100.

ical pregnancy was defined as the presence of an intrauterine gestational sac by transvaginal ultrasonography. The ethical review board of our university approved the study protocol.

Granulosa cell isolation

Granulosa cells collected from follicular fluid after oocyte retrieval procedures was pooled in a tube and centrifuged at 500 *g* for ten minutes. After centrifuging, supernatant was removed, cumulus cells were then fixed in 2.5% glutaraldchyde dissolved in 0.1M phosphate buffer for two hours at room temperature for electron microscopic evaluation. After washing three times for ten minutes in 0.1M phosphate buffer (pH 7.4), cells were post-fixed with 1% osmium tetroxide. The cumulus cells were subsequently infiltrated and embedded in araldite (Epon812, EMS) after dehydrating in an ethanol gradient at room temperature. Semi-thin (one-µm thick) sections for light microscopy were stained with toluidine blue-azure II before examination by light microscopy. An ultracut UCT-R ultramicrotome was used to cut blocks. The ultrathin (70 nm thick) sections were double stained with uranyl acetate and lead citrate, before viewing using a JEOL-1400 electron microscope operating at 80 kV [7].

Statistical analysis

Shapiro-Wilk test was used to test the assumption of normality of groups. Mann-Whitney U test was used as appropriate statistical comparison because of the groups were not distributed normally ($p < 0.05$). Fisher's exact test was used to compare categorical variables.

Data are expressed as median (interquartile range). Differences were considered significant when $p < 0.05$. Box plots were achieved with MS Excel 2010 software.

Results

Light microscopy observations

Observations were carried out at x100 magnification on three fields randomly chosen by means of a DM 6000B microscope and photographed using a digital microscope camera. Both GnRH agonist and antagonist-treated groups showed the presence of two morphologically different cell populations: normal and abnormal cells. Totally, 100 cells per sample were counted as abnormal or normal cells.

In light microscopic evaluation; normal cells had rounded euchromatic nuclei and homogenous cytoplasm. Cytoplasm was stained lightly with toluidine blue. Abnormal cells had smaller and denser nuclei than normal ones. Cytoplasm of abnormal cells were also stained darker than normal cell's cytoplasm. Lipid droplets were observed both in normal and abnormal cells (Figure 1).

Ultrastructural observations

Normal and abnormal cell populations were also characterized detailed with transmission electron microscopy. Cells displaying round, euchromatic nucleus with normal perinuclear cisternae, were identified as normal. These cells had well-developed endoplasmic reticulum and mitochondria with tubular cristae as well as number of lipid droplets in their cytoplasm (Figure 2).

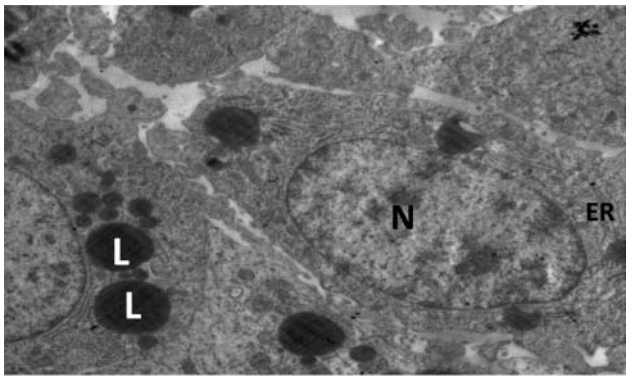

Figure 2. — Ultrastructure of nucleus (N), endoplasmic reticulum (ER), lipid dropletes (L) in normal granulosa cells. Uranyl acetate and lead citrate, x7,000.

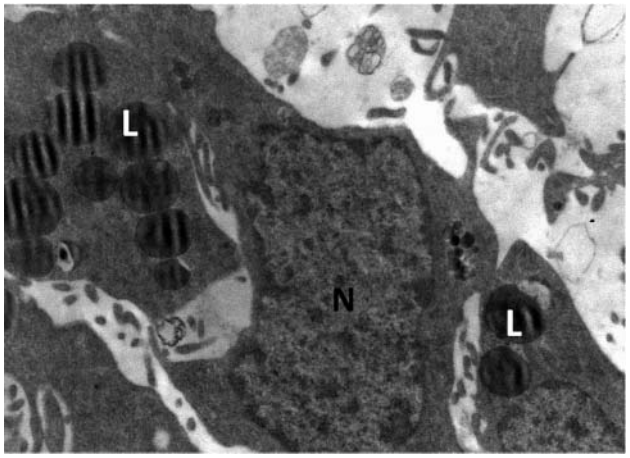

Figure 3. — Ultrastructure of nucleus (N) and lipid dropletes (L) in abnormal granulosa cells. Uranyl acetate and lead citrate, x12,000.

Abnormal cells were characterized as shrunken cells with heterochromatic nuclei. Although the perinuclear cisternae were normal, the contour of the nuclei were irregular. The cytoplasm of these cells were examined condensed and poor in organelles. The ratio of cytoplasm and nucleus were changed. The volume of cytoplasm was decreased (Figure 3).

Both in normal and abnormal cells in agonist and antagonist group were similar in ultrastructural level. In agonist and antagonist group, normal granulosa cells had euchromatic nucleus with normal cytoplasmic organelles. Also abnormal granulosa cells of both groups had shrunk cytoplasm with poor organelles and heterochromatic nuclei with irregular contour. In some of the normal and abnormal aspirated granulosa cells of both agonist and antagonist groups had still intercellular junctions.

Statistical results

When comparing the two groups of women under investigation, the mean percentage of abnormal cells was similar between them (0.0679 ± 0.08977 *vs* 0.0481 ± 0.05164; $p > 0.05$). The mean percentage of normal cells was also similar between agonist and antagonist group (0.9321 ± 0.08977 *vs* 0.9519 ± 0.05164; $p > 0.05$) (Figure 4).

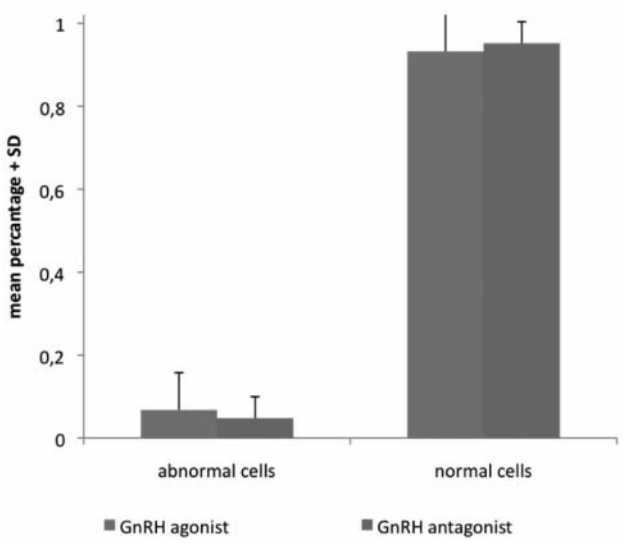

Figure 4. — Distribution of abnormal and normal cell populations in agonist and antagonist groups. Values are expressed as mean percentage ±SD.

Therapeutic outcome parameters

Patient and cycle characteristics, fertilization rates, embryo data and clinical outcomes of the two groups under investigation are shown in Table 1. All parameters investigated were similar between the two stimulation protocols ($p > 0.05$).

GnRH agonist and antagonist therapy was found to have no effect in terms of abnormal granulosa cell percentage (0.0679 ± 0.08977 *vs* 0.0481 ± 0.05164; $p > 0.05$), fertilization [85 (45-90) *vs* 75 (64-93)] and pregnancy rate (37% *vs* 46%).

Discussion

Granulosa cells surrounding the oocytes support oocyte development in several ways. They provide nutrients for the growing oocyte and they produce hormones. They also control both nuclear and cytoplasmic maturation of the oocyte selected for ovulation. It is also known that numerous gap junctions have been observed among the granulosa cells [8] and between the oocyte and the granulosa cells [9]. These gap junctions enable the passage of nucleotides, amino acids and sugars from granulosa cells to the oocyte for its growth and development [10]. Cellular changes in granulosa cells could influence oocyte quality during follicular development and also after the ovulation [11]

In the present study, the authors aimed to compare the percentage of normal and the abnormal granulosa cells and their effect on fertilization and pregnancy rate between GnRH agonist and antagonist treatment. They also detailed the changes in granulosa cell morphology and ultrastructure by light and electron microscopy.

There are studies that report the morphological and ultrastructural properties of granulosa cells, relation between the granulosa cell apoptosis and assisted reproductive technology outcome. Most of the studies focused on apoptosis as cellular injury. In some them, the overall cellular changes in granulosa cells were investigated. Giampietro *et al.* [8] found that granulosa cells apoptosis were comparable between GnRH agonist and antagonist therapy [12]. Another study revealed that fewer apoptotic granulosa cells in women who had an ongoing pregnancy after IVF treatment than in women who did not conceive [13]. Nakahara *et al.* have shown that lower incidence of apoptotic bodies in individual follicles is associated with better outcomes for oocytes [14]. Another study from Nakahara group also revealed that the incidence of apoptotic bodies was significantly higher in mural granulosa cell masses than in cumulus cell masses. They concluded that the incidence of apoptotic bodies in mural granulosa cell masses could be used as an indicator of IVF success [15]. A study by Rotmensch *et al.* described two different granulosa cell morphology in IVF patients. Cells associated with non-fertilizable oocytes had significantly smaller cell areas, tended to be tightly packed, and exhibited abundant intercellular gap junctions and adherence junctions, whereas cells associated with fertilized oocytes tended to be widely dispersed, frequently contained interiorized gap junctional elements and showed morphological correlates of high steroidogenic activity [16]. Ultrastructural properties of human granulosa cells were also studied by Krajci *et al.* in 1989. In this study, early and late preluteinized granulosa cells were characterized by the presence of high amount of pale and homogeneous lipid droplets, moderate accumulation of glycogene, and progressive development of smooth endoplasmic reticulum [17].

Morphometric and ultrastructural analysis of human granulosa cells after GnRH agonist or antagonist was compared by Centurione *et al.* in 2010 [18]. They described two morphologically distinct granulosa cell populations, defined as large/pale and small dark cells. A significantly higher percentage of large/pale cells was detected in the agonist-treated women, whereas the percentage of small/dark cells was significantly higher in antagonist treated group. Their ultrastructural observations showed a typical round and euchromatic nucleus, well-represented smooth endoplasmic reticulum (SER), a number of osmiophilic granules of various size and electron density, as well as of polymorphic mitochondria with tubular cristae in large/pale cells. They also described small/ dark cell ultrastructure as shrunken cell with an indented and heterochromatic nucleus, numerous free ribosomes, scarce SER, and only a few mitochondria exhibiting condensed cristae [18].

The present authors studied overall morphological and ultrastructural changes in granulosa cells under two different stimulation protocols in assisted reproductive technology patients similar with Centurione *et al.*'s study [18]. However the present results were not consistent with this study. The present findings suggest that there was no difference in terms of the percentage of normal and abnormal granulosa cells between GnRH agonist and antagonist treated groups. Morphological and ultrastructural findings were also similar for normal cells of agonist and antagonist treated groups, as well as abnormal cells. In conclusion, both GnRH agonist and antagonist treatments for ovarian stimulation may have similar effects on granulosa cells at morphological and ultrastructural level, as well as on fertilization and pregnancy rates.

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