Abstract. Background: We investigated the effects of the gonadotropin-releasing hormone (GnRH) agonist triptorelin as well the GnRH antagonist cetrorelix on the viability and steroidogenesis in human granulosa luteinized (hGL) cell cultures. Materials and Methods: The hGL cells were obtained from 34 women undergoing ovarian stimulation for IVF treatment. The cells were cultured for 48 h with or without 1 nM or 3 nM of cetrorelix or triptorelin in serum-free media. The cell viability was evaluated by the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay. The concentrations of estradiol and progesterone in culture supernatants were measured by ELISA. Results: Treatment with triptorelin slightly increased cell viability, whereas treatment with 3 nM cetrorelix led to a significant decrease. Estradiol concentrations were reduced with 3 nM triptorelin. Cultures treated with high-dose of either cetrorelix or triptorelin tended to secrete less progesterone than controls. Conclusion: Cetrorelix significantly reduces the viability of hGL cells. Triptorelin and cetrorelix may have minor effects on steroidogenesis. These results suggest that GnRH analogues may influence ovarian functions.

In addition to its central action, it is suggested that GnRH exerts peripheral actions since the presence of GnRHR has been demonstrated in many extrapituitary tissues, including the human ovary. In particular, GnRHR mRNA was detected in human granulosa-lutein cells (hGL) and in ovarian surface epithelial cells, as well as in ovarian cancer cells, where it has been suggested that locally-produced GnRH plays an autocrine/paracrine role (1).

After the discovery of the chemical structure of the GnRH molecule (2), a plethora of GnRH analogues were promptly developed, initially agonists, and later, antagonists. GnRH analogues are widely used by patients undergoing assisted reproductive techniques (ART) in ovarian stimulation protocols in order to achieve lower cancellation rates and higher pregnancy rates. They suppress the endogenous gonadotropin release and prevent the premature luteinizing hormone (LH) surges, which can cause premature ovulation. GnRH agonists, upon binding to the GnRHR cause gonadotropin release (flare-up effect) and their further administration leads to the cessation of secretion through a desensitization mechanism (3-5). The antagonistic analogues directly suppress gonadotropin release by a competitive blocking of the GnRHR (4, 6). The use of GnRH antagonists in ART has been associated with the absence of the flare-up effect, shorter stimulation protocols and a low incidence of ovarian hyperstimulation syndrome (6). However, it has been suggested that GnRH antagonist protocols are associated with slightly lower clinical pregnancy and embryo implantation rates, and with lower estradiol (E₂) levels on the day of ovulation induction, as compared to GnRH agonist protocols (7, 8).

The finding of putative functional GnRHR in the human ovary raised concerns as to the possible effects of GnRH analogues on ovarian functions during ovarian stimulation. Thus many studies were conducted to evaluate the actions of GnRH agonists on ovarian steroidogenesis, mainly in hGL cells.

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cells, but with highly contradictory results, suggesting either inhibition, stimulation, or no effects. Such controversy might result from differences in the methodologies utilized and in the type of GnRH agonists used (9-19). On the other hand, limited studies have been performed to examine the effects of GnRH antagonists on ovarian steroidogenesis (18-22).

Thus there is some controversy about the direct effects of GnRH analogues on the human ovary and hGL cells. Furthermore many researches have investigated the direct effects of GnRH analogues on apoptosis and proliferation predominantly in human ovarian cancer cells, where it is suggested to mediate antiproliferative and proapoptotic actions (23, 24).

The aim of this study was to investigate in vitro the effects of the GnRH agonist triptorelin, as well as those of cetrorelix on the viability of hGL cells. Furthermore, we examined their effects on the steroidogenesis of hGL cells.

Materials and Methods

Granulosa cell collection. The study was conducted at the Laboratory of Physiology, Democritus University of Thrace, Alexandroupolis, Greece with the assistance of the Department of Gynecology and Obstetrics, University Clinic of Schleswig-Holstein, Campus Lübeck, Germany. Granulosa cells were collected at the time of oocyte retrieval from 34 women (27 to 40 years old) who participated in IVF cycles because of male factor infertility. Controlled ovarian hyperstimulation (COH) followed a GnRH antagonist/recombinant follicle-stimulating hormone (rFSH) and a human menopausal gonadotropins (hMG) regimen (Lübeck Protocol), as described elsewhere (19). The study protocol was approved by the Scientific Committee of the University Hospital of Alexandroupolis.

Cell preparation. After collection of oocytes, the follicular fluids were centrifuged at 200 xg for 10 min to separate the granulosa cells. The pellet was washed twice with phosphate-buffered saline (PBS) without Ca²⁺ and Mg²⁺. The cell pellet was resuspended in PBS and transferred to a 15 ml centrifuge tube (Falcon; BD-Biosciences, Heidelberg, Germany). The remaining erythrocytes were removed by incubation in haemolysis buffer (150 mM NaCl, 10 mM NaHCO₃, 0.1 mM EDTA) for 5 min at room temperature. The cells were then pelleted by centrifugation (200 xg for 10 min) and resuspended in culture medium (18).

Cell culture. The collected hGL cells were cultured for 48 h in RPMI-1640 with glutamine (Invitrogen, Karlsruhe, Germany) enriched with 6% fetal calf serum (FCS; Invitrogen), 2% Ultraser G (CytoGen GmbH, Sinn, Germany), 100 IU/ml penicillin and 100 ng/ml streptomycin (Invitrogen), at 37°C in a humidified atmosphere of 5% CO₂.

Considerable attention has been attributed to the fact that high amounts of exogenous gonadotropins are used in COH, which might affect the GnRHR expression. Therefore the cells were cultured in their initial state for 48 h to allow recovery from any effect of the in vivo exposure to gonadotropins, and thus theoretically the effects of IVF hormones were minimised. After this period, the cells were separated and counted with trypan blue exclusion test. They were then seeded at a density of 20,000 live cells/well into 96-well plates and treated for a further 48 h with GnRH analogues. During this period, the culture medium contained no FCS to avoid potential interactions with growth factors present in serum.

Experimental design. The cultures derived from each patient were divided into four treatment groups and a control group. Cultures were treated with the GnRH antagonist cetrorelix (Cetrotide; Serono Europe Ltd., Frankfurt, Germany) and with the GnRH agonist (Gonapeptyl; Ferring, Kiel, Germany) each at concentrations of 1 nM and 3 nM. In the control group, no GnRH agonist or antagonist was added. Each group contained culture wells in such way that at least two cultures from each patient were included in all five groups. Accordingly comparisons were made between cultures belonging to different groups but obtained from the same woman.

After treatment, the viability was evaluated using the [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] (MTT) assay. In addition, the supernatants of each group were collected and stored at −20°C for determination of estradiol (E₂) and progesterone concentrations by enzyme-linked immunosorbent assay (ELISA).

Cell viability count by MTT colorimetric assay. The MTT test was originally described by Mosmann (25) and evaluates the viability of cells. This method is based on the ability of the yellow watersoluble tetrazolium salt MTT to be reduced from the succinate-tetrazolium reductase system belonging to the mitochondrial respiratory chain of metabolic active cells, to the purple, water-insoluble formazan crystals. The quantity of formazan produced is directly proportional to the number of metabolically active cells. The concentration of the created formazan can be determined by measuring the optical density (OD) at 595 nm (26-28).

The procedure used for the MTT assay was as follows: MTT (Sigma, Deisenhofen, Germany) was dissolved at a concentration of 5 mg/ml in sterile PBS and filtered. Then 10 μl of MTT were added to each well with 100 μl of Dulbecco’s medium without phenol red (Sigma-Aldrich, Deisenhofen, Germany) and the plates were incubated at 37°C for 4 h. Subsequently, 100 μl of solvent buffer containing 10% Sodium dodecyl sulphate (SDS) and 50% N,N-dimethylformamide (pH=4.7) were added. The plate remained for 10-20 h in the dark at room temperature. The OD was measured using an ELISA plate reader (MRX: Dynatech Labs., Chantilly, VA, USA) at a wavelength of 595 nm and with a reference filter of 650 nm.

Steroid hormone assays. E₂ and progesterone levels in the media from hGL cell cultures were assayed by commercial ELISA kits according to the manufacturer’s instructions. E₂: Diametra DKO003 (Diametra, Foligno, Italy); sensitivity=10 pg/ml; intra-assay coefficient of variation (CV)=3.69%; inter-assay CV=3.87%. Progesterone: DRG ELISA EIA 1561 (DRG Instruments GmbH, Marburg, Germany); sensitivity=0.045 ng/ml; intra-assay CV=5.4%-6.86%; inter-assay CV=5.5%–9.96%.

Data analysis, statistical methods. At least two cultures from every woman were included in every experimental group. Thus the experimental groups were interdependent, and the comparisons between them were performed with the Wilcoxon’s matched pairs test. p-Value <0.05 was considered statistically significant. All calculations were performed using the Statistica 7.1 software (StatSoft Inc., Tulsa, OK, USA).
**Results**

*Effects of triptorelin and cetrorelix on cell viability.* The cell viability measurements are presented in Table I. Treatment of cultured cells with 3 nM or 1 nM triptorelin induced a slight increase of 10% or 4% respectively, of cell viability compared to untreated cells. Treatment with 3 nM cetrorelix for 48 h led to a 28% decrease of cell viability in comparison with the control group (*p*<0.005). Exposure to 1 nM cetrorelix had no significant effects compared to the untreated control group (*p*>0.05). In addition the differences that were found among the experimental groups were statistically significant (*p*<0.05). In particular the decrease of the cell viability induced by the high dose of cetrorelix (3 nM) compared with the slight increase induced by triptorelin (3 nM, 1 nM) or the low dose of cetrorelix (1 nM) was statistically significant. The increase in viability between the low dose of cetrorelix (1 nM) and triptorelin (1 nM) was also statistically significant.

*Effects of triptorelin and cetrorelix on steroidogenesis.* Exposing hGL cells either to triptorelin or to cetrorelix for 48 h did not affect significantly the E2 secretion, compared to control values, although E2 levels from cultures treated with 3 nM of triptorelin were lower than controls. Statistical significant lower E2 levels were observed in cultures treated with 3 nM triptorelin compared to 1 nM cetrorelix-treated cells (Table II).

Regarding the progesterone production, no statistically significant differences were found between cells treated with GnRH analogues and cells incubated with the culture medium only. Nevertheless, treatment with 3 nM of cetrorelix or triptorelin reduced the progesterone levels by 19% and 10% respectively, but not in a statistically significant way. Cell cultures treated with 3 nM of cetrorelix produced statistically significant lower amounts of progesterone than cultures treated with 1 nM of cetrorelix and those treated with 1 nM of triptorelin (Table II).

**Discussion**

The results of the present study show that cetrorelix significantly reduces and triptorelin slightly increases the viability of hGL cells. High amounts of triptorelin reduced the E2 production of hGL cells compared to cells treated with low doses of cetrorelix. Furthermore, cultures treated with 3 nM cetrorelix or triptorelin tended to secrete lower levels of progesterone.

In a previous study comparing granulosa luteinized cell cultures treated either with cetrorelix or with the GnRH agonist leuprorelide, we found that the GnRH analogues did not significantly affect steroidogenesis (19). It is worth noting that the present results derived from hGL cell cultures under basal conditions. Special care was given in order to avoid the presence of growth factors in the culture media. With the presence of gonadotropins or other growth factors which might be present in cultures media or supplement as FCS, the effects of GnRH analogues on the function of granulosa luteinized cells could be different. It has been reported that GnRHR causes transactivation of tyrosine kinase receptors (RTKs), such as epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGFR) and fibroblast growth factor receptor (FGFR) (29). In cases where ligands for RTKs are present, the cross-talk between GnRHR and RTKs might be responsible for effects much different from those observed in the present study.

The observed reduction in viability by a high dose of cetrorelix was considerable and in line with the observed reduction of progesterone production in cultures treated with a high dose of cetrorelix. Regarding their antiproliferative effects, GnRH antagonists can induce apoptosis in certain ovarian cancer lines by mediated GnRHR activation of Gi protein (30, 31).

Can the observed effects be attributed to the whole spectrum of GnRH agonists and GnRH antagonists? In our opinion, it is not self-evident that other GnRH agonists or antagonists will necessarily give similar results. It has been suggested that “different GnRH ligands, both agonists and antagonists, can...
determine preferential interactions with different intracellular protein complexes through stabilization of the GnRHR in different conformations, leading to the activation of specific signal transduction pathways (30). The classical intracellular GnRH signalling pathway, which is also predominant in gonadotrope cells, involves the activation of Gq protein, which leads to the activation of phospholipase Cβ (PLCβ), generation of inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) as well as to the downstream mobilization of intracellular Ca2+ and protein kinase C (PKC). The other possible signal transduction pathway of GnRHR is via Gs, to activate adenylyl cyclase and elevate cAMP (30, 31). Moreover, binding of GnRH agonists or antagonists to GnRHR may stabilize it in a conformation which activates the Gi protein, leading to cell cycle arrest and to induction of apoptosis (30, 31).

Furthermore, it has been reported that GnRHR signal transduction interacts with other receptors such as EGFR and FGFR (30, 31). Thus, it is reasonable to presume that the cross-talk between GnRHR and EGFR or FGFR could be different according to the type of agonistic or antagonistic ligand binding the GnRHR. In other words, not all GnRH agonists or antagonists will generate exactly the same response to binding GnRHR of hGL cells. In the study of Bussenot et al. (12), five GnRH agonists were compared regarding their effect on E2 production from hGL cell cultures, with results showing that some GnRH agonists increased E2 production, whereas others did not. Much of the discrepancy on the reported controversial effects of GnRH analogues on hGL cells may lie on the use of different GnRH analogues.

Another interesting point is the dose of GnRH analogues. It has been suggested that the action of GnRH and GnRH agonists on cancer cell lines is dual and biphasic: low concentrations stimulate cellular growth, migration and invasion, whereas high concentrations inhibit these functions (29). It is not clear if this dual and biphasic action is valid for hGL cells regarding their metabolic and steroidogenic activity. Previous studies with unstimulated hGL cell cultures have provided indications for a dose-dependent effect on steroidogenesis (9, 13). However, results from other studies do not support this (32-34). The present study provides indications for a dose-dependent effect of the GnRH antagonist cetrorelix on cell viability and progesterone production. Apparently the finding of reduced viability is new and interesting but on its own it cannot provide a strong argument for modification of current IVF protocols. This result can be translated as a thought for the optimization of IVF outcomes with stronger luteal support. On the other hand, the GnRH agonist triptorelin seemed to have a dose-dependent negative effect on E2 production of hGL cell cultures.

In conclusion, the present results support the notion that GnRH analogues influence the viability and steroidogenesis of hGL cells suggesting that they may have direct ovarian effects. Further experiments are required in order to elucidate the underlying mechanisms.

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References


