Long-term effects of GnRH agonist, GnRH antagonist, and estrogen plus progesterone treatment on apoptosis related genes in rat ovary

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Objectives: To define the long-term effects of GnRH antagonist, GnRH agonist, and estrogen plus progesterone treatments on apoptosis and apoptosis-related gene expressions, including bcl2, bax, and cyt c in rat ovary.

Design: Prospective placebo-controlled experimental study.

Setting: Obstetrics and Gynecology and Medical Biology and Genetics university departments.

Interventions: Forty female wistar rats that were 3 to 4 months of age.

Main Outcome Measure(s): Assessment of morphology, histology of ovaries, determination of the number of apoptotic cells, and analysis of apoptosis-related gene expression of bcl2, bax, and cyt c in the rat ovaries.

Results: Long-term GnRH antagonist treatment significantly increased bax gene expression, but the ratio of bcl2:bax gene expression was constant compared with control group. The GnRH agonist treatment significantly increased cyt c gene expression, and estrogen plus progesterone treatment significantly decreased bcl2 and significantly increased cyt c expressions. In the estrogen plus progesterone group, ovaries were cystic and larger than in the other groups. There was no significant morphologic change between the other groups.

Conclusions: Long-term administration of GnRH agonist, GnRH antagonist, and estrogen plus progesterone can modulate the apoptosis-related genes in rat ovary. Although GnRH antagonist treatment does not influence apoptosis, GnRH agonist and estrogen plus progesterone treatments seem to influence apoptosis in rat the ovary. Further clinical studies focusing on the effect of these agents on apoptosis-related genes could be performed.

Key Words: GnRH agonist, antagonist, bcl2, bax, cyt c, rat model

Follicular atresia is regulated by endocrine, paracrine, and genetic factors. Gonadotropins and estrogens serve as survival factors, whereas androgens induce apoptosis in follicles (1, 2). The intracellular mechanism responsible for follicular atresia is still unclear. The bcl-2 protein family, bcl-2 and bax, has been shown to regulate apoptosis in the ovary (3, 4). Bax causes apoptosis by inducing permeabilization of mitochondrial membranes and opening of mitochondrial porin channels (5). Bcl-2 is capable of blocking the induction of apoptosis, first at the mitochondrial level by forming heterodimers with bax, and second after bax-induced pore formation (5–7). Increase in the bcl2:bax ratio protects the cell from apoptosis, whereas a decrease in the ratio determines the induction of apoptosis (8). In transgenic mice, excessive expression of bcl-2 leads to decreased follicular apoptosis and atresia (9). Supporting the crucial role of this protein family in the regulation of apoptosis in the ovary, bax-deficient mice have abnormal follicles with an excessive number of granulosa cells (10). Cytochrome c (cyt c), being a proapoptotic factor situated in the outer membrane of mitochondrion is a crucial structure in the apoptotic pathway. Release of cyt c is a universal event during apoptotic cell death.

Gonadotropin-releasing hormone (GnRH) agonists and antagonists have been widely used in the treatment of gynecologic diseases, such as endometriosis, uterine fibroids, dysmenorrhea, and hormone-dependent tumors. The GnRH agonists have immediate decline in gonadotropin levels, with an immediate therapeutic effect within 2–72 h. When the ovarian stimulation regimen with either GnRH
agonist or GnRH antagonist is not successful, or pregnancy can not be achieved, these agents are given for repetitive cycles. Experimental studies have been done to find out the effect of these agents on ovarian apoptosis. One study has found that in rats treated with leuprolide acetate, Bcl-xL expression was decreased and apoptotic indices were increased (11). Another in vitro study showed that both GnRH agonist and GnRH antagonist increased apoptosis in rat ovary (12). Oral contraceptive use suppresses FSH concentrations; therefore, it was hypothesized that long-term high-dose oral contraceptive use would postpone menopause to later age. Whereas some investigators found a delaying effect of oral contraceptive use on menopause age (13), others found no effect (14–16).

GnRH agonist and antagonist are used in assisted reproductive technology programs and hormone-related gynecologic conditions, not only for one cycle, but for repetitive cycles. Despite numerous studies, the long-term effect of GnRH agonist and antagonist on ovarian apoptosis is still unknown. The purpose of the present study was to clarify the long-term effect of GnRH agonist, GnRH antagonist, and estrogen plus progesterone treatment on apoptosis and related genes in rat ovary.

MATERIALS AND METHODS

Animals

The study was performed using 3–4-month-old Wistar albino rats weighing 200–250 g. The animals were locally bred and were maintained under controlled condition (20–23 °C, lights on from 05:00 to 19:00). All protocols were reviewed and approved by the Dokuz Eylul University Animal Ethics Committee. Vaginal smears were taken, and only animals displaying the same estrus cycle were taken into the study. Each group consisted of 10 rats, being kept in cages containing 5 rats each. Group 1 (control) was given placebo (normal saline) every day by gastric lavage. Group 2 (GnRH agonist) received leuprolide acetate in depot form (Lucrine Depot; Abbot, IL) 1 mg/kg every 30 days, the dose given in another animal study (17). Group 3 (GnRH antagonist) received cetrorelix (Cetrodite; Serono, Geneva, Switzerland) 0.1 mg/kg every 2 days, the dose as calculated according to the bioavailability of the drug in rats (18). Group 4 (estrogen plus progesterone) was given estradiol valerate and norethisteron enantate in depot form (Mesiygna; Schering, Berlin, Germany) 0.5 mg/kg every 30 days, the dose used in toxicologic studies of this drug (19).

The animals were killed by a toxic dose of ether after 60 days. Under sterile conditions laparatomy was performed. For immunohistochemistry and apoptosis assays, one of the ovaries from each animal was fixed at 4°C in buffered 10% formaldehyde and embedded in paraffin wax. The other ovary was put in 5-mL sterile tubes containing RNA stabilization and protection solution (RNAlater; Qiagen, Hamburg, Germany).

Bcl-2 and Bax Immunohistochemistry

The blocks were sectioned onto poly-L-lysine–coated slides. The avidin-biotin-peroxidase method was performed, using the primary monoclonal antibodies against bcl-2 protein (1:100, Clone 10C4; Bioscience, CA), Bax protein (1:100; Clone 6A7; Biosource International, CA). The sections were deparaffinized in xylene, rehydrated, and immersed in distilled water, and endogenous peroxidase activity was blocked using a 0.3% solution of hydrogen peroxidase in phosphate-buffered saline (PBS). After antigen retrieval by heating in 10 mmol/L citrate buffer (pH 6.0), all three primary antibodies were applied for 60 min at room temperature and washed in PBS. Biotinylated secondary antibodies and streptavidin-peroxidase complex (Dako Corp, Copenhagen, Denmark) were added consecutively for 10 min at room temperature and washed in PBS. The peroxidase activity was visualized with 0.03% 3,3’-diaminobenzidine tetrahydrochloride (Sigma Chemical, St. Louis, MO) applied for 5 min. After rinsing in distilled water and counterstaining in hematoxylin, the slides were dehydrated and mounted. A semiquantitative scoring system was used for both bax and bcl-2 staining. In this scoring system, the degree of positive staining in follicular cells is evaluated by scoring on a scale of 0 to 4 for intensity (I) and for distribution (D). Tissues with 1 × D ≤ 4 were considered weakly positive, and those with >4 were strongly positive. Should the calculated number be 0, the sample is evaluated as negative (20).

Apoptotic Assays

Slides of 5 μm thickness were obtained from the ovarian tissue, fixed at 4°C in buffered 10% formaldehyde, and embedded in paraffin wax. Samples were deparaffinized and treated with PBS containing 0.2 mg/mL saponin (Sigma, Taufkirchen, Germany) and 20 μg/mL proteinase K (Roche, Mannheim, Germany) for 20 min. Slides were rinsed with distilled water and heated in 50% formamide (Sigma, Taufkirchen, Germany) prewarmed to 56°C for 20 min. Next, slides were transferred into ice-cold PBS for 5 min, blocked in 3% nonfat dry milk, and incubated with mAb F7-26 (10 μg/mL PBS containing 1% nonfat dry milk). For each ovary, 1,000 cells in follicles were counted. Apoptotic index was calculated by counting cells having the specific features of condensed chromatin staining per 1,000 cells in the follicle. Additionally, apoptotic index was evaluated by counting 1,000 cells on hematoxylin-eosin–stained sections under light microscope in at least five randomly selected high-power fields (×400) and expressed as the number of apoptotic cell per 100 cells. The morphologic criteria for apoptotic cells/apoptotic bodies used in the present study were as follows: 1) a single round mass with condensed strongly eosinophilic cytoplasm with a single clump of strongly basophilic material representing chromatin condensation; 2) similar to the first structure but with condensed chromatin fragmented into more than one piece; and 3) fragments of condensed chromatin material without surrounding cytoplasm. The apoptotic cells/apoptotic bodies most frequently appear as single structures.

RNA Extraction

One of the ovaries from each animal was used for RNA extraction. The ovary was immediately put into RNA stabilization
and protection solution (RNAlater). Total RNA was isolated from samples stored in RNA stabilization and protection solution using RNeasy Mini Kit (Qiagen). All RNA preparations were stored at −80°C until use.

Reverse-Transcription PCR Total RNA (4 μg) was reverse transcribed in a final volume of 20 μL with 20 μg random hexamer and 200U MuLV (Promega, Madison, WI), according to the manufacturer’s guidelines. Aliquots corresponding to 200 ng RNA were then amplified in polymerase chain reaction (PCR) buffer containing, 200 μmol/L dNTPs (Promega), 25 pmol each primer (MWG Biotech AG, Ebersberg, Germany), and 1 U Taq polymerase (MBI Fermentase) in a final volume of 25 μL.

Aliquots of the same cDNA were amplified with bcl2, bax, and cyt c primers. The sequences of the oligonucleotide primers for each gene obtained from cDNA sequences registered at Genebank do not share significant sequence homology with other genes, as analyzed by BLAST search. The sequences of oligonucleotide primers for each gene as shown in Table 1 were used for reverse-transcription (RT) PCR (Table 1). Each amplification was carried out for 30 cycles, an annealing at 58°C, and an extension at 72°C for 1 min, and extension at 72°C for 1 min, according to the manufacturer’s guidelines. Aliquots corresponding to 200 ng RNA were then amplified in polymerase chain reaction (PCR) buffer containing, 200 μmol/L dNTPs (Promega), 25 pmol each primer (MWG Biotech AG, Ebersberg, Germany), and 1 U Taq polymerase (MBI Fermentase) in a final volume of 25 μL.

The PCR products were electrophoretically resolved on 2% agarose–TBE gel containing 0.5 μg/mL ethidium bromide and run for 1.5 h at 8 V/cm. The gel image was captured using the Eagle Sight Software (Stratagene) system, and the RT-PCR bands were analyzed with Molecular Analyst software (Bio-Rad) after scanning with a Bio-Rad GS700 imaging densitometer. For semiquantitative analysis, mRNA of the housekeeping gene HPRT was coamplified using the same RT-PCR conditions as for each experiment. Bcl2 and bax mRNA levels were expressed as the ratio of signal intensity of all experimental bands for the target genes in relation to that for the coamplified HPRT.

### Statistical Analysis

The RT-PCR results were analyzed by Kruskal-Wallis and Mann-Whitney tests, and immunohistochemical and apoptotic assays results were analyzed by chi-squared test. A P value of <0.05 was considered to be significant for all analyses.

### RESULTS

#### Animals

All animals in the control (group 1) and GnRH agonist (group 2) groups were healthy during the 2 months of the study. In group 3 (GnRH antagonist), one, and in group 4 (estrogen plus progesterone), two of the animals died during the experiment, and all statistical analyses were done using the final number of animals in each group. In addition, during the experiment, the animals in group 4 lost their body hair and their color went pale. Only in group 4 was a significant change observed during laparotomy, all of the ovaries removed from these animals were cystic and larger in size compared with the other groups. In the other groups, no significant morphologic change was observed in the animals or in their ovaries.

#### Bcl-2 and Bax Immunohistochemistry and Apoptotic Assays

In the estrogen plus progesterone group, the number of apoptosis-positive cells and cells representing chromatin condensation in hematoxylin-eosin–stained sections were significantly higher (P=0.015). No significant differences between groups were observed for bcl-2 and bax immunohistochemistry (P=0.182 and P=0.346, respectively).

#### RT-PCR

The expressions of mean values of bcl-2, bax, and cyt c genes among groups are given in Figures 1 and 2. The results of mRNA expression of examined factors are presented as changes in the target gene expression, normalized to housekeeping gene HPRT.

| Primer sequences and product sizes of amplified genes. |  |
|---|---|---|
| **Gene** | **Sequence (5’→3’)** | **PCR product size (bp)** |
| Bcl2 | ACCTGACGCCCTTCAC | 168 |
| | AGGTACTCACTGATCCAC | 212 |
| Bax | AAGAAGCTGAGCGAGT | 227 |
| Cyt c | TGGGTGATGTTGAGAAAGG | 227 |

The RT-PCR bands were analyzed with Molecular Analyst software (Bio-Rad) after scanning with a Bio-Rad GS700 imaging densitometer. For semiquantitative analysis, mRNA of the housekeeping gene HPRT was coamplified using the same RT-PCR conditions as for each experiment. Bcl2 and bax mRNA levels were expressed as the ratio of signal intensity of all experimental bands for the target genes in relation to that for the coamplified HPRT.


<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’→3’)</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT</td>
<td>GTGGAGATGATCTCTCTCACT</td>
<td>262</td>
</tr>
<tr>
<td></td>
<td>ACATGATTCAATCCTCAGAG</td>
<td>168</td>
</tr>
<tr>
<td>Bax</td>
<td>AAGAAGCTGAGCGAGT</td>
<td>212</td>
</tr>
<tr>
<td>Cyt c</td>
<td>TGGGTGATGTTGAGAAAGG</td>
<td>227</td>
</tr>
</tbody>
</table>
0.38 and 0.18, respectively, showing that cell survival was negatively affected in this group (Table 2). Compared with group 1, the decrease in bcl-2 and increase in cyt c were statistically significant (P < .001 and P < .001, respectively).

In summary, bcl-2 mRNA expressions was down-regulated in groups 3 and 4, but not affected in group 2, and the expression of cyt c mRNA was highest in the estrogen plus progesterone group and was significantly up-regulated after long-term treatment.

**DISCUSSION**

Our data demonstrate that long-term GnRH antagonist treatment increases proapoptotic bax gene expression, and that estrogen plus progesterone treatment decreases bcl-2 expression and increases cyt c expression. When we evaluate the ratios of bcl-2 gene to proapoptotic gene bax expression, GnRH antagonist treatment does not seem to change the ratios compared with the control group. However, GnRH agonist and especially estrogen plus progesterone treatments seem to negatively affect this ratio to favor apoptosis. This effect was more clear in the estrogen plus progesterone group. The loss of body hair on the animals and cystic formations in the ovaries in this group may indicate a generally increased apoptosis with this treatment. In an animal model, it has been shown that estrogen-treated mice showed retarded progression to anagen hair development and irregular patchy hair pattern, and in addition to this, estrogen antagonist–treated animals displayed rapid anagen development with diffuse hair growth pattern compared with the control animals (21). In a recent study, it was shown that supraphysiologic estrogen exposure results in cyst formation in ovaries of adult rats, with disturbed folliculogenesis, early maturation of oocytes, and increased apoptosis (22). These results are similar to our results, suggesting an increase in cell turnover with altered apoptosis-related gene expressions. Long-term treatment with oral contraceptives has been questioned by human studies, and there are conflicting results (13–16). The debate is about whether oral contraceptive use can delay menopause or not, or can even accelerate it. A well designed cohort study in humans suggested that long-term users of high-dose oral contraceptives experience menopause at a slightly younger age compared with woman who did not use oral contraceptives or used only low-dose oral contraceptives (23).

There are conflicting data about the effect of GnRH agonist and antagonist treatment in mammalian ovaries. Cyclophosphamide-treated rhesus monkeys given GnRH agonist compared with control animals that were not given GnRH agonist had a higher number of primordial follicles (24). Paborrell et al. have shown that whereas GnRH antagonist decreased apoptosis in rat follicles, GnRH agonist leuproplide acetate increased apoptosis (25). On the other hand, Durlinger et al. suggested that GnRH antagonist treatment induces atresia in preovulatory follicles in rat (26). A recent study demonstrated an acute depletion in murine primordial follicle reserve induced by Cetrotide, a GnRH antagonist (27). The present data indicate that the ratio of antiapoptotic gene bcl-2 to proapoptotic gene bax and cyt c was not affected by GnRH antagonist treatment compared with control. Additionally, GnRH agonist and estrogen plus progesterone treatments decreased the ratio of antiapoptotic gene bcl-2 to proapoptotic gene bax and increased cyt c expression compared with control. This effect was more obvious in the estrogen plus progesterone group. Giampietro et al. compared the levels of apoptosis, but not apoptosis-related genes, in granulosa cells from woman undergoing ART cycles treated with either GnRH agonist or GnRH antagonist; apoptosis was detected in both groups, but they were not distinguishable from each other (28). It is obvious that GnRH antagonists and agonists affect the apoptotic processes in mammalian ovaries, but the clinical importance of the issue is not clear. Granulosa cell apoptosis has been related to ART outcome by some investigators; lower granulosa cell apoptosis was found in women who had ongoing pregnancy after IVF treatment than in

![Image](image_url)
women who did not (29), and lower incidence of apoptotic bodies in follicles was associated with better outcomes for oocytes (30). The present results showing the long-term effect of GnRH antagonist and agonist on rat ovary could be applied to clinical trials to determine whether GnRH antagonist and agonist have an effect on apoptosis-related genes in ART cycles.

In conclusion, our data show the modulatory effects of GnRH antagonist, GnRH agonist, and estrogen plus progesterone treatment on apoptosis-related genes in rat ovary. Whereas long-term GnRH antagonist treatment did not influence the expression of proapoptotic bax and cyt c genes, GnRH agonist and estrogen plus progesterone treatments influenced the expression of these proapoptotic genes. It is important to note that these observations are for rat ovary, and to apply these observations to other species requires further studies.

REFERENCES

5. Shimizu S, Narita M, Tsujimoto Y. Bcl-2 family proteins regulate the release of apoptotic cytochrome c by the mitochondrial channel VDAC.

FIGURE 2

Agarose gel showing RT-PCR products Cyt c genes representative one sample. Lanes 1: control group; lanes 2: GnRH agonist group; lanes 3: GnRH antagonist group; lanes 4: estrogen plus progesterone group. Mean Cyt c expressions among groups. *P<0.01 vs. control, +P>0.01 vs. control.


