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

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Objective: 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.
Animal(s): Forty female wistar rats that were 3 to 4 mounths of age.
Intervention(s): Forty rats were randomly divided into 4 groups of 10 each. In group 1 (control) each rat received normal saline as placebo by gastric lavage. In group 2 (GnRH agonist) 1 mg/kg leuprolide acetate in depot form was given for 30 days. In group 3 (GnRH antagonist) each animal received 0.1 mg/kg cetrorelix every 2 days. In group 4 (estrogen plus progesterone) 0.5 mg/kg estradiol valerate and norethisteron enantate in depot form was given every 30 days. After 60 days, the animals were killed.
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.
Result(s): 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 progesteron treatment significantly decreased bcl 2 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.
Conclusion(s): 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 angonist 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. (Fertil Steril® 2009;91:2006–11. ©2009 by American Society for Reproductive Medicine.)

Key Words: GnRH agonist, antagonist, bcl 2, 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 bcl-2: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 first stimulate and then suppress gonadotropin secretion through desensitization and receptor down-regulation, and for this reason they have been used in assisted reproductive technology (ART) programs. The GnRH antagonists, unlike the agonists, produce an immediate decline in gonadotropin levels, with an immediate therapeutic effect within 2–72 h. When the ovarian stimulation regimen with either GnRH

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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-x_L 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 (Cetrotide; 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 phospate-buffered saline (PBS), 0.01 mol/L, pH 7.5. 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 deionized water and counterstaining in hematoxylin, the slides were dehydrated and mounted. A semiquantitative scoring system was used for both bax and bcl-2 straining. 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 $I \times D \leq 4$ were considered weakly positive, and those with >4 were strongly positiveShould 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 deparafinized 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 transfered 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 ($\times$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