Short-term Administration of Gonadotropin-Releasing Hormone Agonist (Buserelin) Induces Apoptosis in Rat Ovarian Developmental Follicles

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ABSTRACT

Objective: The aim of the present study was to investigate the effects of buserelin on the development of follicles, apoptosis index, and steroid hormones level.

Methods: Twenty four 3 month old female rats were randomly divided into three groups, that is, a low dose group, a high dose group and a control group (n=8). Buserelin and normal saline were injected subcutaneously for five days. Thirty days after the first injection, the ovaries were removed for staining. Moreover, blood samples were collected and centrifuged. Their serum was used for measuring estradiol and progesterone levels using Enzyme-Linked Immunosorbent Assay (ELISA).

Results: The findings revealed a significant decrease in the mean of secondary and graffian follicles in the high dose group as compared with the control group (P=0.037, P=0.034, respectively). The serum estradiol level increased significantly in the high dose group as compared with the low dose and control groups (P=0.027, P=0.047, respectively). The serum progesterone level decreased, although not significantly. In contrast to the control group, the significant increase of apoptotic cell death was found in primordial, unilaminar and multilaminar follicles in high dose group (P=0.004, P=0.049, P=0.047, respectively).

Conclusion: The findings of this study suggest that short-term administration of high dose buserelin increases the serum estradiol level and apoptosis in the granulosa cells but has an inhibitory effect on follicular development.

Keywords: GnRH agonist, TUNEL, ELISA, Follicle, Ovary, Rat

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INTRODUCTION

The Gonadotropin Releasing Hormone (GnRH) is the brain factor that plays an essential and effective role in reproductive function. GnRH is released in a pulsatile mode into the hypophyseal portal circulation. It then reaches the putative aim on gonadotrope cells in the anterior pituitary (1). The reproductive function is mediated by GnRH binding to G Protein Coupled Receptors (GPCR), the stimulation of the synthesis, and the release of the pituitary gonadotropins, Luteinizing Hormone (LH), and Follicle-Stimulating Hormone (FSH) (2).

Continuous stimulation, rather than pulsatile stimulation, of pituitary GnRHR by administered GnRH agonists desensitizes and down-regulates GnRHRs (3). GnRH agonists are delivered in a concatenated form to turn off reproductive function by inhibiting gonadotropin construction (4). In the ovary, GnRH has been shown to elicit a mix of both inhibitory and stimulatory responses affecting the ovarian function (5). The development of ovarian follicles is evaluated to be regulated by diverse factors such as gonadotropins (6, 7), steroid hormones (8), cytokines (9), and growth factors (10). Moreover, different gondal functions such as folliculogenesis, steroidogenesis, and apoptosis are in turn regulated by gonadotropins (11). Programed cell death or apoptosis has been considered a biological procedure through which unwanted cells are deleted in response to developmental signals or a toxic stimulus.

The major characteristics of apoptosis are DNA fragmentation, cell shrinkage, plasma membrane blebbing, and apoptotic bodies formation (12). Apoptosis also plays the main role in the process of maintaining the reproductive system (13). During growth and development of human ovarian follicles, only a small number of adjacent follicles proceed to the ovulatory stage, whereas more than 99% of follicles undergo the apoptotic process of atresia (14). Granulosa cells protect the growing oocyte until ovulation and also produce hormones related to oocyte

maturation and ovulation (15). Many researchers have studied factors that directly or indirectly regulate apoptosis of granulosa cells. Recent studies have considered a physiological role for GnRH in granulosa cell apoptosis. When it comes to this study, the aim was to evaluate the effects of GnRH agonists (Buserelin) on follicular growth, apoptosis, and changes in levels of estradiol and progesterone in adult female rats.

MATERIAL AND METHODS

Animals

All procedures on rats were performed according to the 'Principles of Laboratory Animal Care' (NIH publication no. 85–23, revised 1985), as well as the specific rules of the 'Animal Care and Use Committee', National Medical and Health Service System. Twenty four 3 month old mature female rats were included in this study. All rats were allowed to feed *ad libitum* and were kept in a light (12 h light/12 h dark) and temperature (22 to 24 °C) controlled room.

To assess the similarity of the estrous cycle phases, vaginal smears were taken. Sterile cotton swabs were soaked in distilled water to enter the vagina and were rotated on the vaginal wall. Vaginal epithelial cells were placed on slides, dried and fixed with 70% ethanol. Samples were stained according to the papanicolaou's method. Stained vaginal smears were observed under a light microscope for the identification of estrous cycle phase according to specifications cells. Estrous cycle phase of rats was metestrus (Figure 1).

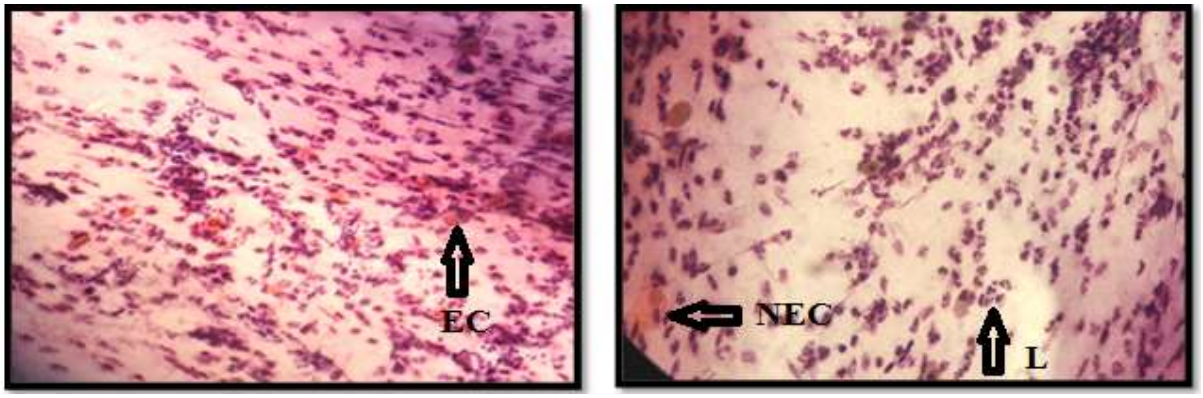

Fig 1: Photomicrographs of stained vaginal smear from female rats at metestrus. Leukocytes (L), Nucleated Epithelial Cell (NEC), Enucleated Cell (EC).

Chemicals

Buserelin acetate (Injection 1mg/ml, Suprefact®) was purchased from a local distributor. All chemicals were prepared from Merck (Darmstadt, Germany) or Sigma-Alderich (Munich, Germany). *In Situ* Cell Death Detection Kit™ was purchased from Roche (Rotkreuz, Switzerland). Kits were used to measure estradiol and progesterone (Monobind Inc. Lake Forest, USA).

Experimental procedure

24 adult wistar rats were randomly divided into three groups (n=8). Rats were treated with 300 µg/kg buserelin (SC, low dose group), 600 µg/kg buserelin (SC, high dose group) and normal saline (SC, control group) for five days at specific times. Thirty days after the first injection, rats were anesthetized with choloroform and their ovaries were dissected out.

Morphometric study

Ovary fragments were routinely processed and embedded in paraffin. The stained sections by hematoxylin and eosin were observed under a light microscope. The number of different types of follicles [primordial, primary, secondary, graffian follicle] and corpus luteum were counted by two independent expert examiners in six serial sections of each rat ovary. Follicles are classified according to the definitions below:

Primary oocyte that surrounded by a single layer of the flattened cells called primordial follicles. The follicle is called unilaminar primary when a simple cuboidal epithelium environs the primary oocyte. The oocyte in multilaminar primary follicle surrounded by a stratified follicular epithelium. When the small spaces created within this epithelium, follicles called secondary. The spaces developed and graffian follicle shows a large single antrum (16).

Measurement of circulating levels of steroid hormones

Blood samples were collected from the heart and centrifuged at 170g for 15 min. The serum was isolated and levels of estradiol (E2) and progesterone were measured adopting an Enzyme-Linked Immunosorbent Assay (ELISA) method.

Tunel

The rate of apoptosis in ovarian was evaluated in 5 μm thick formalin fixed paraffin embedded tissues of ovary using the TUNEL kit according to the manufacture´s instructions.

The numbers of TUNEL-positive cells were determined by counting them in the granulosa cells. All morphometric measurements were carried out by at least two independent expert examiners blindly.

Statistical analysis

The statistical analyses were performed using GraphPad Prism 6 version 6.01 for windows, GraphPad Software. The results are presented as mean ± SEM, and $P < 0.05$ is considered significant.

RESULTS

The development of follicles was examined using adult rats and H&E staining (Figure 2). Values represent the number of follicles counted in ovaries from the low dose, high dose and the control groups. The means of secondary and graffian follicles for high dose and control groups were 8.29 ± 3.38, 24.86 ± 5.09 and 4 ± 1.31, 12.67 ± 4.1, respectively, significant decreased (P=0.037, P=0.034, respectively). In contrast, the mean of the primordial, unilaminar and multilaminar primary follicles, and the corpus luteum did not show significant changes compared to the control group (Figure 3).

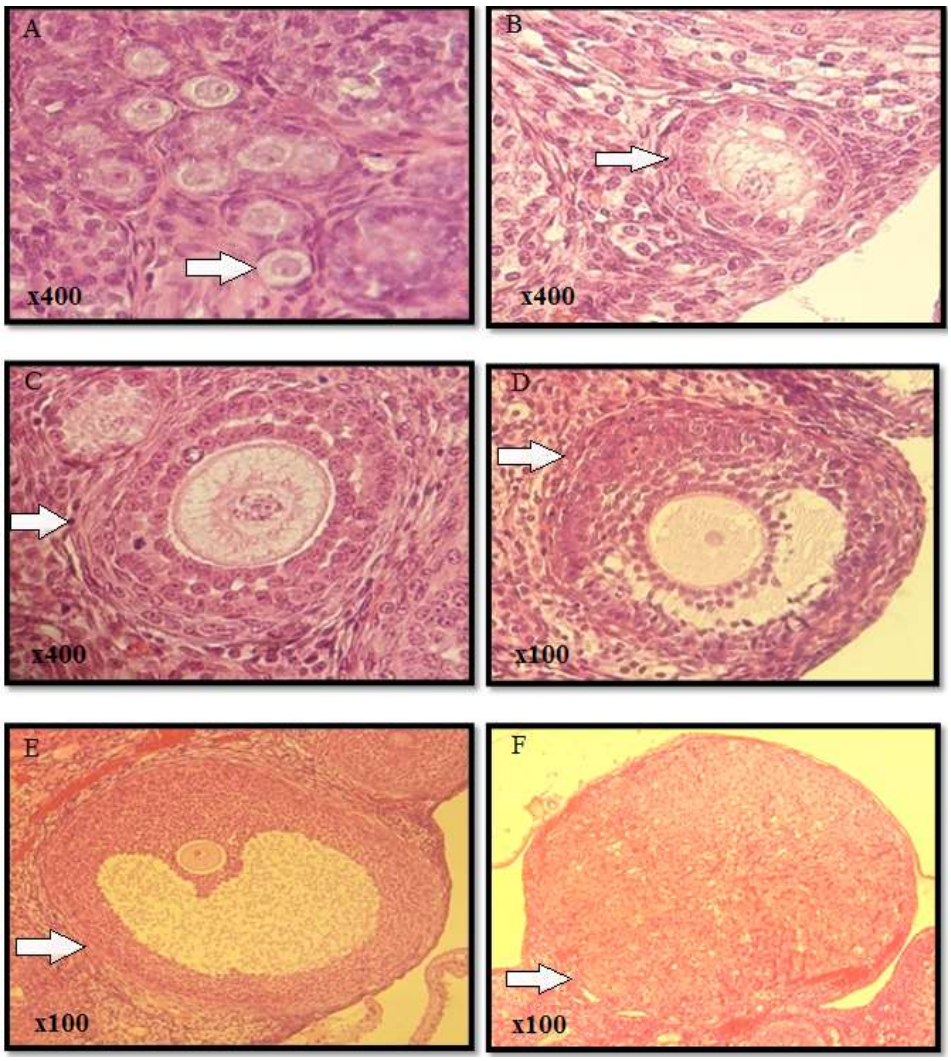

Fig 2: Photomicrographs of different types of follicles (H&E staining); Primordial (A), Unilaminar primary (B), Multilaminar primary (C), Secondary (D), Graffian (E) follicles and corpus luteum (F).

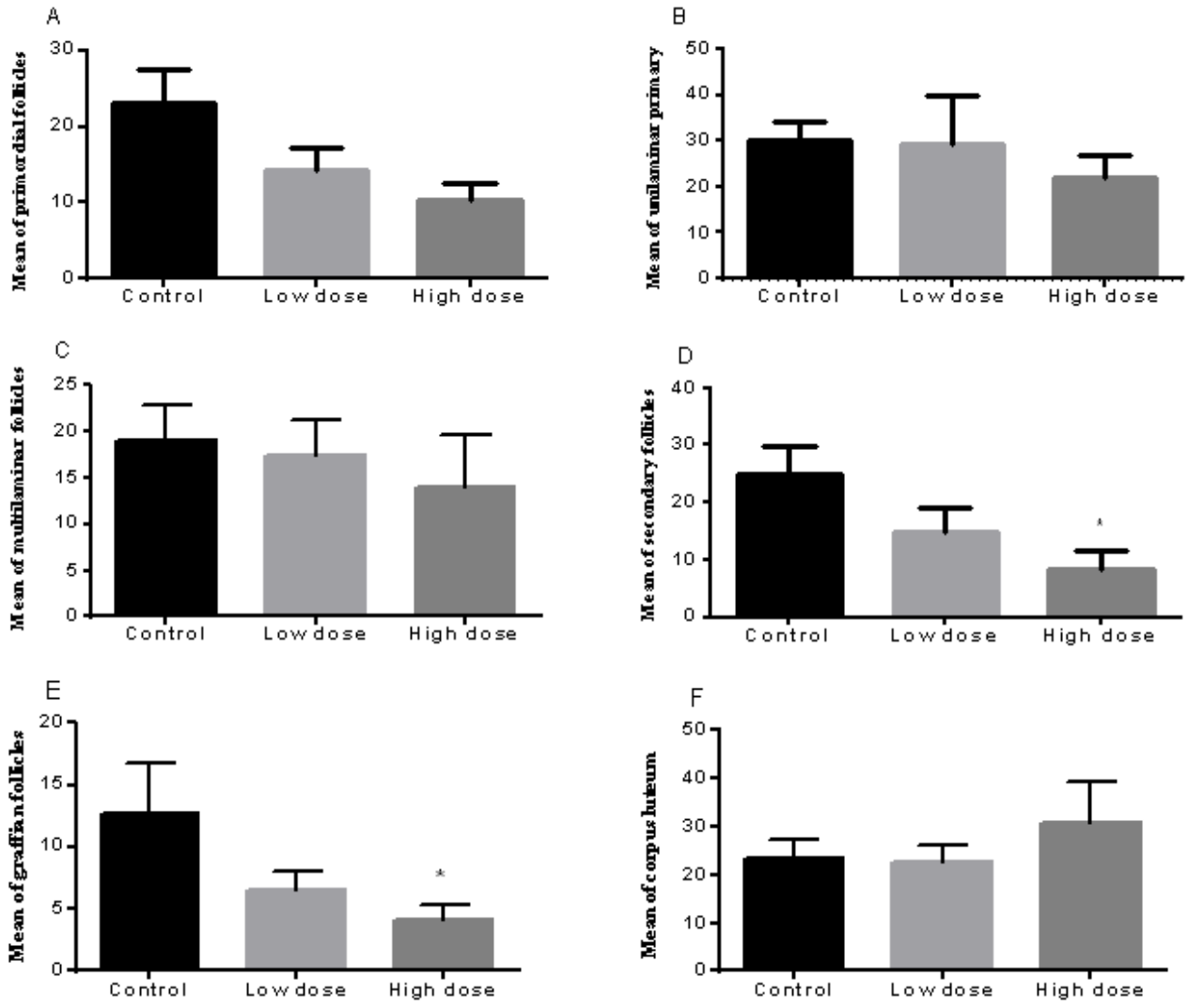

Fig 3: Effect of invivo treatment of different doses of buserelin on development of follicles in the ovaries of rat. Comparison mean number of primordial (A), unilaminar primary (B), multilaminar primary (C), secondary (D), graffian (E) follicles and corpus luteum (F) in different groups.

Estradiol (E2) and progesterone levels were measured using an Enzyme Linked Immunosorbent Assay (ELISA) kit, according to the manfacture's instructions (Monobind. USA). The findings revealed that buserelin increased significantly the serum estradiol (E2) level in the high dose group compared with the control and low dose groups (Low dose group: 47.29 ± 13.45 pg/ml,

High dose group: 165.31 ± 37.08 pg/ml, Control group: 58.98 ± 22.68 pg/ml, P=0.027, P=0.047, respectively), whereas serum progesterone levels decreased, though not significantly (Figure 4).

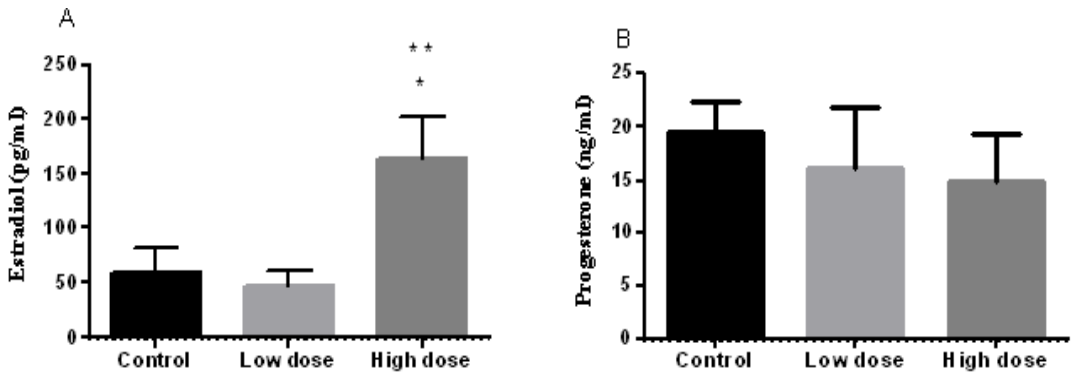

Fig 4: Concentration of estradiol (A) and progesterone (B) in serum of adult female rat after short-term buserelin (GnRH-Ag) administration. Data represent the average values, the concentration of estradiol increased in the high dose group versus the control group. * $P < 0.05$.

Percentages of apoptotic cell death were evaluated by in situ cell death detection kit^{TM} and TUNEL-positive cells in follicles of treated groups were compared with those of the control rats (Figure 5).

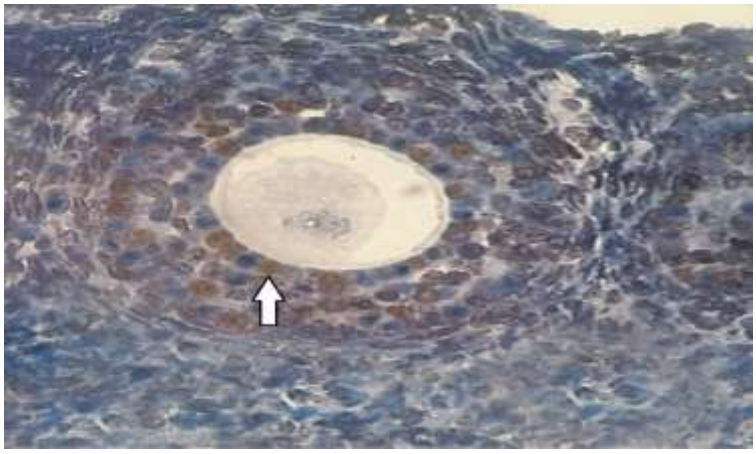

Fig 5: Evaluation of apoptosis in ovarian follicles by TUNEL assay. Multilaminar primary follicle (400x). Apoptotic cells are indicated by arrows.

The percentage of apoptotic cells increased significantly in primordial follicles in low and high dose groups (P=0.015, P=0.004), unilaminar follicles in the high dose group (P=0.049) and multilaminar primary follicles in the high dose group (P=0.047) compared with those in the control group, respectively. However, no significant changes in TUNEL-positive apoptotic cells were observed in secondary and graffian follicles (Figure 6).

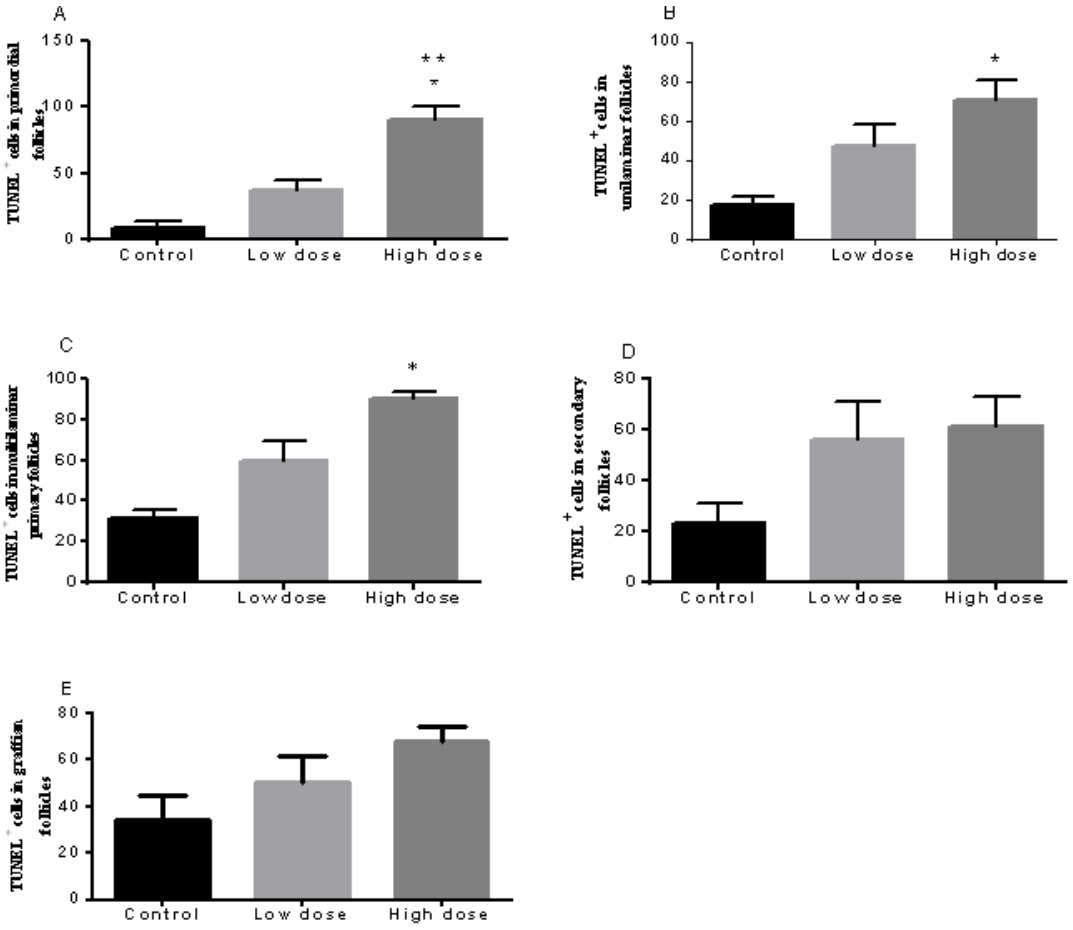

Fig 6: The percentage of apoptotic cells with different doses of buserelin was assessed by the TUNEL. Data points represent the mean ± SEM. * $p< 0.05$, statistically significant. A: Primordial follicle, B: Unilaminar primary follicle, C: Multilaminar primary follicle, D: Secondary follicle, E: Graffian follicle.

DISCUSSION

Today, various methods for treatment of infertility are available; one of these methods uses synthetic GnRH agonists. The direct effects of GnRH or its agonists on the ovary have been reported in previous research (17, 18). Buserelin is one of the GnRH agonists with two substitutions that mimic the function of the hypothalamic-releasing hormone (19).

Granulosa cells play a major role in the selection, development, and support of the ovarian follicles and ovarian cycle through the control of oocyte maturation and production of steroid hormones, estradiol, and progesterone (20).

Previous studies have shown that treatment with a GnRH agonist in rats reduced the number of growing follicles in the ovaries; however, it did not change the number of Corpus Luteum (CL) (21). A study conducted by Ghanadee et al. (22) found that high doses of GnRH agonist stimulated the primay growth of follicles and inhibited the growth of mature follicles. Moreover, the pulsed discharge of the GnRH increased with aging. Therefore, injecting high doses of GnRH agonist can have more inhibitory effects on follicle growth in younger ages (22). In confirmation of the studies conducted so far, this study found that the number of ovarian follicles in experimental groups decreased compared to that in the control group. This decline in the secondary and graffian follicles was found to be significant. The high dose of GnRH agonists inhibited the development of mature and pre-ovulatory follicles. This might be due to its stimulatory effect on the pituitary to release high levels of FSH and LH at the start of injection, and to inhibit gonadotropin secretion with the negative regulator of gonadotrop receptors with continued injection, resulting in a significant reduction in the rate of growth of mature follicles (secondary and graffian). Non-pulsatile administration of high doses of GnRH agonists inhibits gonadotropins secretion. This inhibitory effect is followed by a negative regulation of GnRH

pituitary receptors resistant to the release of original GnRH (23). However, before it can inhibit the secretion of gonadotropins, it stimulates the pituitary gland, increasing the level of FSH and LH. However, a few days after the onset of agonists GnRH injection, the drop in FSH and LH levels was sharper than that in the LH levels (24). Furthermore, granulosa cells have GnRH receptors and buserelin can directly affect the cells by binding to receptors in granulosa cells and affecting the pituitary-gonadal axis in the process.

GnRH and its agonists have some inhibitory effects on stimulation of the aromatase activity, LH receptor, and biosynthesis of progesterone (7, 25, 26). Along with having an inhibitory effect, GnRH stimulates oocyte maturation (27), ovulation (28), ovarian tissue plasminogen activator gene expression (29), glycolysis (30) and acute steroidogenesis (25). In vivo studies on adult male and female hypophysectomized rats have shown that GnRH have stimulatory and inhibitory effects on gonadal activities, especially steroidogenesis (31). A study conducted in 1998 by Andreu et al., concluded that GnRH agonist reduced the circulating levels of progesterone and storage in collagenase-dispersed ovarian cell cultures, while the production of the estradiol (E2) was increased (31). The results of this study confirm those of previous studies because serum estradiol levels in the treated group with a high dose of GnRH agonist compared to the lower dose and control groups indicates a significant increase. Also, the progesterone was reduced in the groups treated with GnRH agonist, although the reduction was not significant. Apoptosis in primordial, primary unilaminar and multilaminar follicles went through a significant increase, but in secondary and graffian follicles, this increase was reported as not significant. Because of having receptors, mature follicles are more active in production of estradiol, which justifies the enhanced level of estradiol hormone.

In 1994, a study performed by Billig et al. on hypophysectomy immature female rats showed that an increase in the apoptosis in ovarian GnRH agonist was directly dependent on dose and time (20). Buserelin, through gonadotropin releasing hormone receptors, increased apoptosis in ovarian granulosa cells in the ovarian follicles (32). Zhao et al. conducted a study in 2000 and showed that the incidence of apoptosis in cells cultured in humans and pigs was directly increased by buserelin (33).

The results of the study showed that the apoptosis in primordial, unilaminar and multilaminar primary follicles in the second experimental group, i.e. the group with high doses of GnRH agonist, had a significant increase compared to that in the control group, hence confirming the findings from previous studies.

CONCLUSION

In this study, we found that the short-term GnRH-Ag (buserelin) administration decreases the number of follicles in mature rat ovaries. Also, this study shows that the buserelin increases apoptosis in the granulosa cells and serum estradiol (E2) level.

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