ORIGINAL ARTICLE

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

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ABSTRACT

Objective: *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: a low-dose group, a high-dose group and a control group (n = 8). Buserelin and normal saline were injected subcutaneously for 5 days. Thirty days after the first injection, the ovaries were removed for staining. Blood samples were collected and centrifuged. Their serum was used for measuring estradiol and progesterone levels, using enzyme-linked immunosorbent assay.*

Results: *The findings revealed a significant decrease in the mean of secondary and Graafian follicles in the high-dose group compared with the control group (*p = *0.037,* p = *0.034, respectively). The serum estradiol level increased significantly in the high-dose group, 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 multi-laminar follicles in the 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: Gonadotropin-releasing hormone agonist, enzyme-linked immunosorbent assay, follicle, ovary, rat, TUNEL.

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is the brain factor that plays an essential and effective role in reproductive function. It 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, 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 GnRH receptor (GnRHR) by administered GnRH agonists desensitizes and downregulates GnRHRs (3). The 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). Programmed cell death or apoptosis has been considered a biological

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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. The aim of this study was to evaluate the effects of GnRH agonists (buserelin) on follicular growth, apoptosis and changes in levels of estradiol (E2) 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-hour light/12-hour dark) and temperature- controlled room (22°C–24°C).

To assess the similarity of the oestrous 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 method. Stained vaginal smears were observed under a light microscope for the identification of oestrous cycle phase according to specifications cells. Oestrous cycle phase of rats was metestrus (Fig. 1).

Chemicals

Buserelin acetate (injection 1 mg/ml, Suprefact®) was purchased from a local distributor. All chemicals were prepared from Merck (Darmstadt, Germany) or Sigma-Aldrich (Munich, Germany). *In Situ* Cell Death Detection Kit™ was purchased from Roche (Rotkreuz, Switzerland). Kits were used to measure E2 and progesterone (Monobind Inc, Lake Forest, USA).

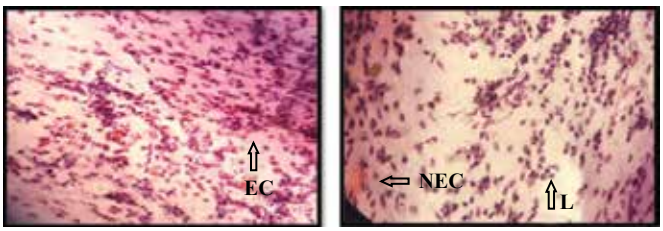

Fig. 1: Photomicrographs of stained vaginal smear from female rats at metestrus. Leucocytes (L), nucleated epithelial cell (NEC) and enucleated cell (EC).

Experimental procedure

Twenty-four 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 5 days at specific times. Thirty days after the first injection, rats were anesthetized with chloroform and their ovaries were dissected out.

Morphometric study

Ovary fragments were routinely processed and embedded in paraffin. The stained sections by haematoxylin and eosin (H&E) were observed under a light microscope. The number of different types of follicles (primordial, primary, secondary, Graafian 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 was 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 multi-laminar primary follicle is surrounded by a stratified follicular epithelium. When the small spaces are created within this epithelium, follicles are called secondary. The spaces developed and Graafian follicle shows a large single antrum (16).

Measurement of circulating levels of steroid hormones

Blood samples were collected from the heart and centrifuged at 170 g for 15 minutes. The serum was isolated, and levels of E2 and progesterone were measured adopting an enzyme-linked immunosorbent assay (ELISA) method.

TUNEL

The rate of apoptosis in ovarian tissues was evaluated in 5 µm-thick formalin fixed paraffin-embedded tissues of the ovary using the terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling (TUNEL) kit according to the manufacturer´s instructions.

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

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 (Fig. 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 Graafian 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, significantly decreased (p = 0.037, p = 0.034, respectively). In contrast, the mean of the primordial unilaminar and multi-laminar primary follicles and the corpus luteum did not show significant changes compared to the control group (Fig. 3).

Estradiol and progesterone levels were measured using an ELISA kit, according to the manufacturer's instructions (Monobind Inc). The findings revealed that buserelin significantly increased the serum 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 (Fig. 4).

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

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 multi-laminar, 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 Graafian follicles (Fig. 6).

DISCUSSION

Various methods for the 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, E2 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* (21). A study conducted by Ghanadee *et al* (22) found that high doses of GnRH agonist stimulated the primary growth of follicles and inhibited the growth of mature follicles. Moreover, the pulsed discharge of the GnRH increased with ageing. Therefore, injecting high doses of GnRH agonist can have more inhibitory effects on follicle growth in

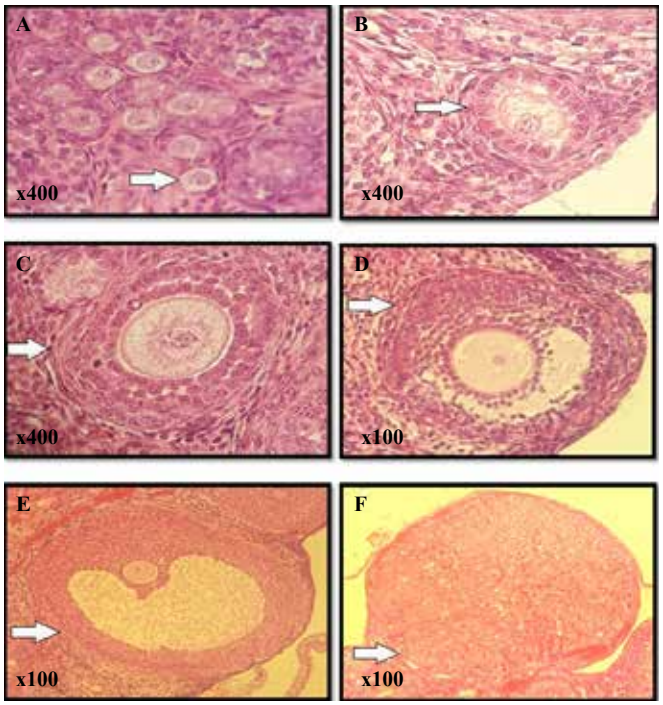

Fig. 2: Photomicrographs of different types of follicles (H&E staining): primordial (A), unilaminar primary (B), multi-laminar primary (C), secondary (D), Graafian (E) follicles and corpus luteum (F).

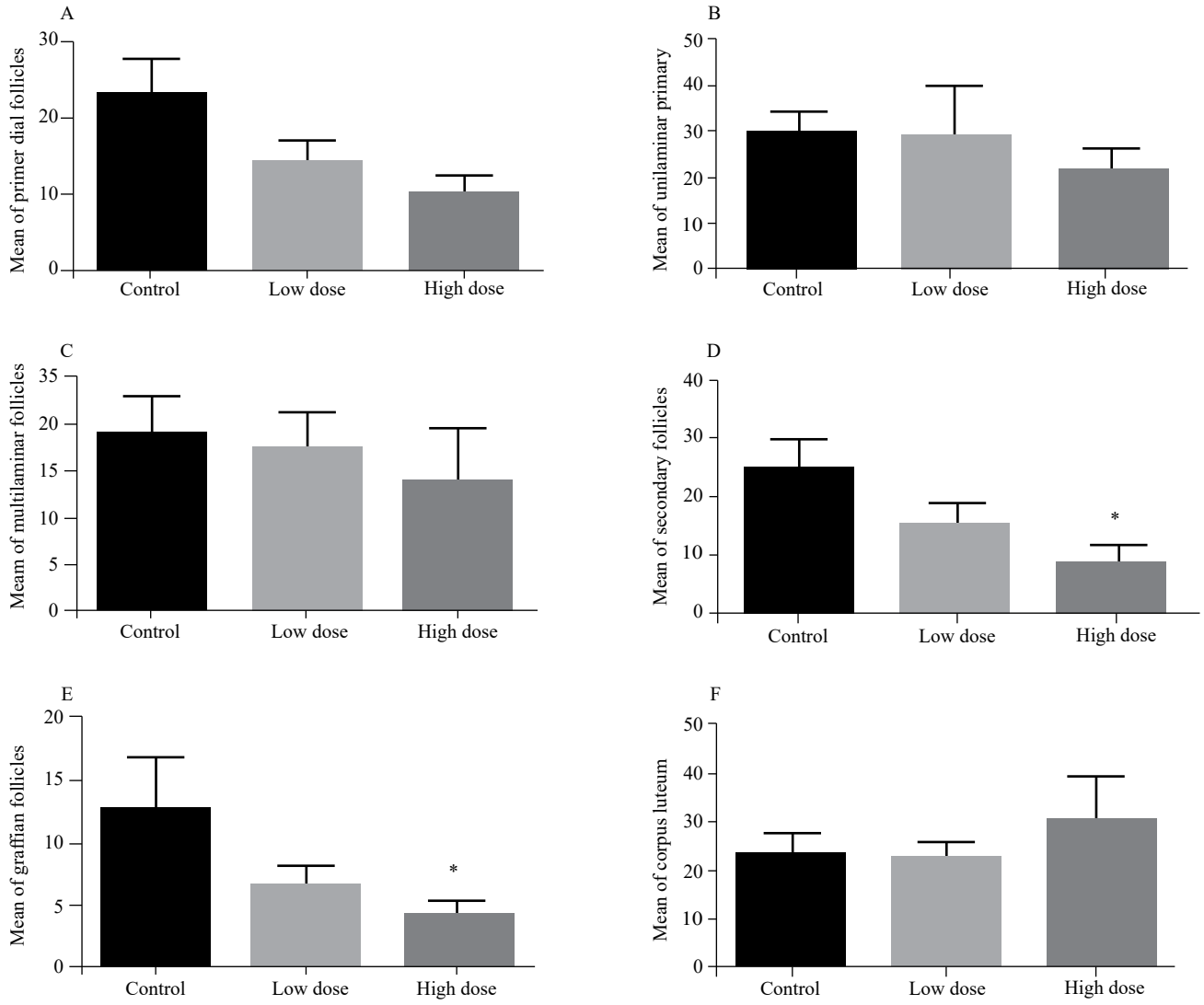

Fig. 3: Effect of *in vivo* treatment of different doses of buserelin on development of follicles in the ovaries of rat. Comparison of mean number of primordial (A), unilaminar primary (B), multi-laminar primary (C), secondary (D), Graafian (E) follicles and corpus luteum (F) in different groups.

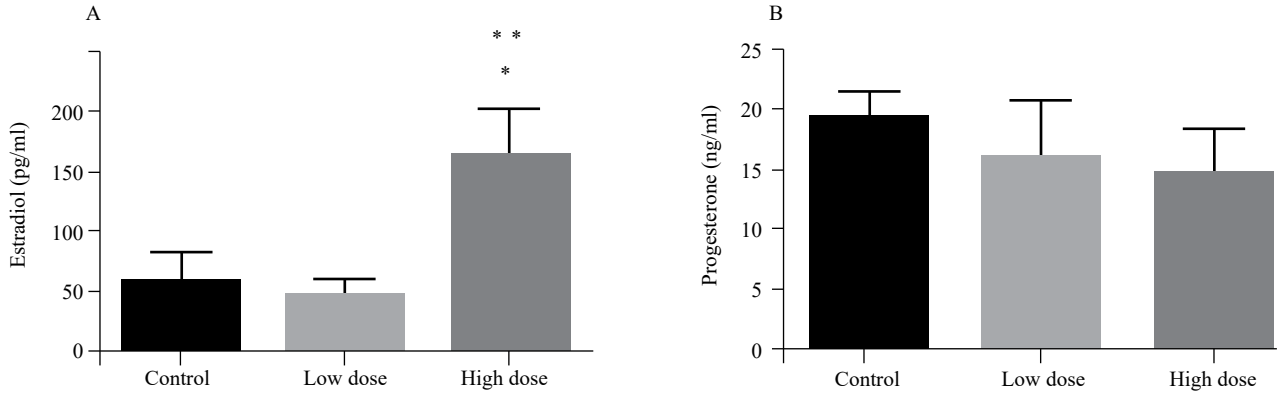

Fig. 4: Concentration of estradiol (A) and progesterone (B) in serum of adult female rat after short-term buserelin (GnRH-Ag) administration. Data represent the average values, the concentration of estradiol increased in the high-dose group *versus* the control group. * $p < 0.05$.

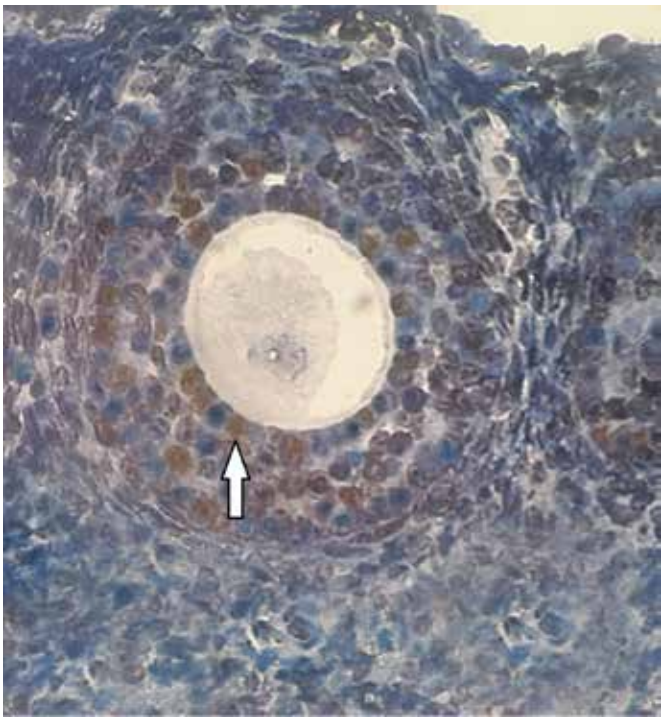

Fig. 5: Evaluation of apoptosis in ovarian follicles by TUNEL assay. Multilaminar primary follicle (×400). Apoptotic cells are indicated by the arrows.

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 Graafian 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 gonadotropin receptors with continued injection, resulting in a significant reduction in the rate of growth of mature follicles (secondary and Graafian). 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.

Gonadotropin-releasing hormone 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 agonists reduced the circulating levels of progesterone and storage in collagenase-dispersed ovarian cell cultures, while the production of the E2 was increased (31). The results of this study confirm those of previous studies because serum E2 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 Graafian follicles, this increase was reported as not significant. Because of having receptors, mature follicles are more active in the production of E2, which justifies the enhanced level of E2 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 multi-laminar primary follicles in the second experimental group, that is, 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 the serum E2 level.

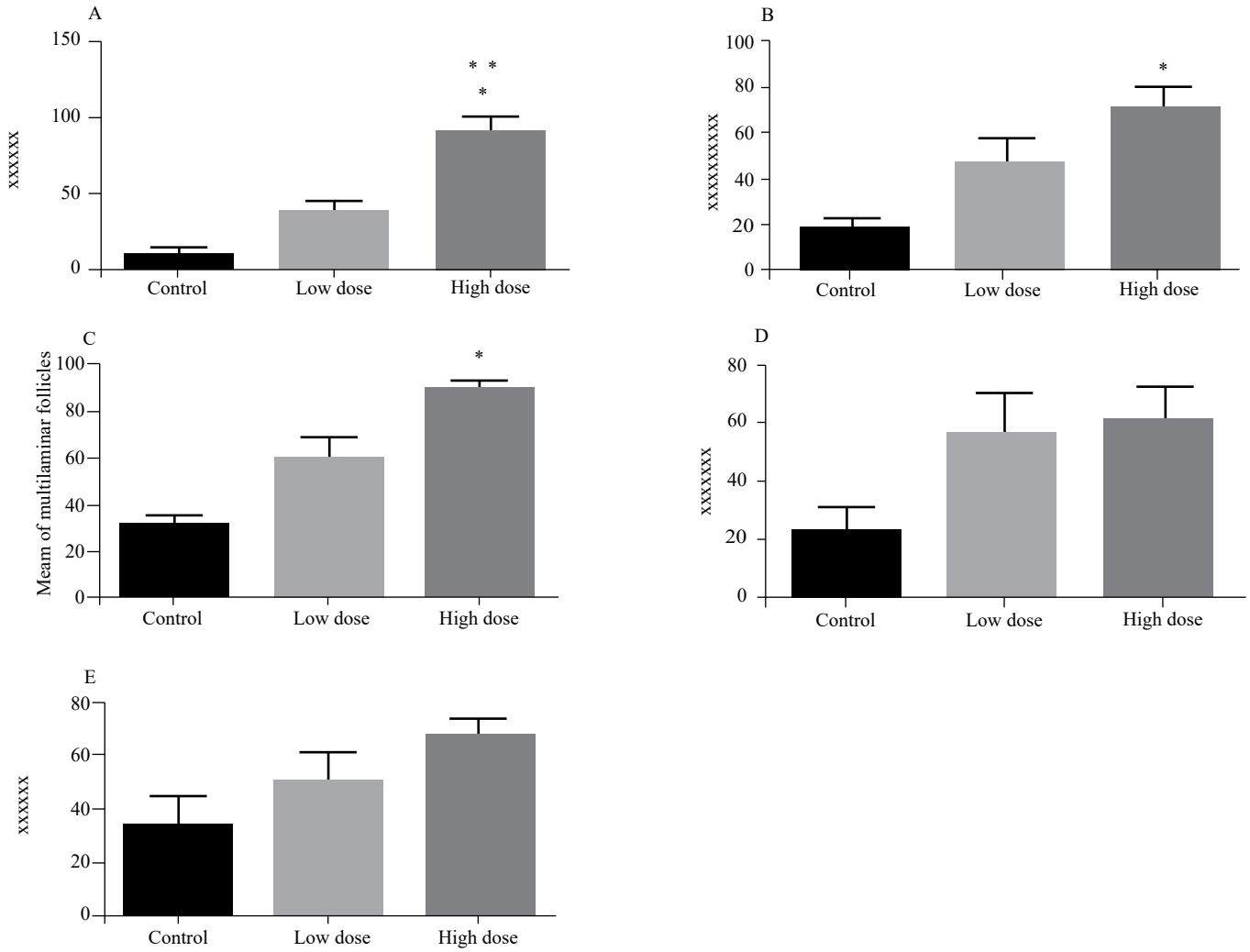

Fig. 6: The percentage of apoptotic cells with different doses of buserelin was assessed by the TUNEL. Data points represent the mean ± SEM. * $p < 0.05$, statistically significant. (A) Primordial follicle, (B) unilaminar primary follicle, (C) multi-laminar primary follicle, (D) secondary follicle, (E) Graafian follicle.

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