Short-term buserelin administration induces apoptosis and morphological changes in adult rat testes

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Abstract

Purpose: To investigate the effect of buserelin on gonadal structure and function in adult male rats.

Methods: Twenty-four adult Wistar male rats were divided into three groups: two treated groups and controls. The first and second treated groups received 300 (low dose) and 500 (high dose) µg/kg buserelin, respectively, and the control group received normal saline. All groups were treated subcutaneously for five days.

Results: The seminiferous tubular epithelial thickness was significantly decreased in the treated groups compared with those in the control. There was a significant increase in apoptotic cell death in high dose treated group compared with low dose treated and control groups. No significant difference in serum testosterone level was observed after one month in the three groups.

Conclusion: Buserelin induces apoptotic cell death and decreased diameter and epithelium thickness of seminiferous tubules in the adult rat testes.

Key words: Gonadotropin-Releasing Hormone. Testosterone. Apoptosis. Testis. Rats.
Introduction

As a very important phenomenon in nature, reproduction is controlled by the hypothalamic-pituitary-gonadal (HPG) axis in all vertebrates. This axis is a blend of both neural and endocrine tissues that function as a coordinator unit in the regulation of fertility. GnRH, a decapeptide, is secreted from the hypothalamus in a pulsatile manner and released to the pituitary gland. In the anterior pituitary gland, the gonadotropic cells act as moderators between the GnRH signal and production of steroid hormone by the gonads. Binding of GnRH to the receptors can start up intracellular events within the gonadotropes. These events lead to the synthesis and release of gonadotropins, Luteinizing hormone (LH) and Follicle-stimulating hormone (FSH). LH stimulates the production of hormones by gonads that play a major role in reproduction. GnRH receptors are locally expressed in the testes, brain, and placenta of mammals.

These receptors were determined in rat gonads from 14.5 to 21.5 days post coitus and it has been revealed that GnRH has paradoxical effects on steroidogenesis in the testis and its inhibitory effects or apoptosis induction occurs only during the late stage of spermatogenesis. Triptorelin, Leuprorelin Acetate and other synthetic agonists of GnRH inhibit characteristics functions of testes while, the stimulatory effects of GnRH agonists like leuprolide resulted in a marked improvement in the number of spermatogenic colonies in the area of recipient seminiferous tubules.

There are two synthetic forms of GnRH included GnRH-agonists and GnRH-antagonists which have clinical applications. buserelin, a gonadotropin-releasing hormone agonist, is a therapeutic drug suppressing the release of LH and FSH. Thus, it is used in the treatment of hormone-responsive cancers such as prostate cancer. Increase in the occurrence of germ cells apoptosis and reduction in testosterone level may be detected in the treatment of prostate cancer with buserelin.

In keeping with our line of research focused on the elucidation of Buserelin related effects on some selected characteristics of the testis, the impact of two different doses of buserelin was investigated in the spermatogenesis of adult rats.

Methods

Protocol of this study was approved by the university ethical committee for animal study (no. ir.umsu.rec.1393.240).

Twenty-four three-month-old Wistar male rats with a body weight of about 200g were used. All rats were allowed to feed ad libitum and were maintained in a light (12h light/12h dark) and temperature (22 to 24°C) controlled room.

Chemicals

Buserelin acetate (Injection 1mg/ml, Suprefact®) was purchased from the local distributor. All chemicals were obtained from Sigma (Canada) or Merck (Darmstadt, Germany). Testosterone assay ELISA kit was purchased from Dia Metra (ITALY). In Situ Cell Death Detection KitTM was purchased from Roche (Germany).

Experimental design

Three-month-old Wistar rats were allotted into three groups (n=8): two treated groups and one control. Treated groups 1 (LDT) and 2 (HDT) received a subcutaneous injection of 300 (low dose) and 500 (high dose) µg/kg buserelin acetate for five days respectively. The controls received a subcutaneous injection of normal saline for five days. One month after the first injection, the testicles were dissected and collected from anesthetized rats.
Testis morphometry

For histological morphometry using a light microscope, one testis of each rat was fixed in Bouin's solution and routinely processed and embedded in paraffin. Subsequently, serial sections of 5 µm were prepared and stained with hematoxylin and eosin and periodic acid-Schiff. At least 60 round or nearly round tubules were chosen randomly and measured for each animal. The epithelium thickness (height) and diameter of seminiferous tubule were assessed in the same tubules. Testis morphometric was executed by light microscope using an ocular micrometer calibrated with a stage micrometer.

Testosterone assay

Blood samples were collected from the rats' hearts between 9:30-15:00. Blood samples were centrifuged at 3000 rpm for 15 minutes and serum was separated and stored at -80°C until analysis. The testosterone level was assessed using enzyme-linked immunosorbent assay (ELISA). Briefly, Twenty-five µl of standards and serum samples of the experiment were added to each well of the ELISA plate. Afterward, 100 µl of the diluted conjugate solution was added to each of these wells. Then, the plate was incubated at 37°C temperature for one hour. Subsequently, the wells were washed twice with 300µl of distilled water and 100µl of the tetramethylbenzidine (TMB) substrate was added to each well. The plate was incubated at room temperature (22-28°C) for 15 minutes in the dark, 100µl of stop solution was added and absorbance was read at 450nm using a microplate reader.

TUNEL staining

The rate of apoptosis in testicular tissues was assessed in 5µm thick formalin fixed paraffin embedded testicular tissues using TUNEL assay. Sections were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol and washed in PBS. Next, proteinase K (30µg/ml) was added to each testis section and incubated for 30 minutes at room temperature. The TUNEL reaction was performed using In Situ Cell Death Detection Kits (Roche) which uses an optimized terminal transferase (TdT) to label free 3’OH ends in genomic DNA with fluorescein-dUTP, followed by detection of incorporated fluorescein by an anti-fluorescein antibody POD conjugate and visualization of the antibody by the immuno-complexed POD with diaminobenzidine (DAB) as a substrate reaction. The sections were incubated with TUNEL reaction mixture in a humidified chamber at 37°C for 60 minutes. After one hour, the sections were covered by anti-fluorescein-POD at 37°C for 30 minutes in a humidified chamber. After each step, the tissue sections were washed twice. Finally, the sections were incubated with DAB solution for 10 minutes at room temperature to stained positive cells brown. The sections were stained with hematoxylin as a background color for light microscopy. In the negative control slides, the terminal deoxynucleotidyl transferase (TdT) enzyme was omitted from the labeling reaction.

Statistical analysis

All data were statistically evaluated by one-way ANOVA and Tukey’s multiple comparison tests. P<0.05 was considered significant. All data are presented as mean±SEM.

Results

Testosterone concentration

Testosterone levels in the treated groups were compared to the control group. The
mean testosterone levels in LTD, HDT, and Control groups were 2.71±0.25ng/ml and 2.12±0.61ng/ml, and 3.14±0.45ng/ml respectively. No significant difference was found in serum level of testosterone among three groups (P>0.05) (Figure 1).

Figure 1 - The effect of buserelin on serum testosterone concentration in adult rats. No significant differences were indicated in the three groups (P=0.327).

Testicular Morphometric

Seminiferous tubule diameter and seminiferous epithelial thickness were assessed in the testicular morphometric. The seminiferous tubules and intertubular space were perceived normal in the control group (Figure 2). In contrast, the germinal cells dissociation, and cell depletion ratio in seminiferous tubule were significantly higher than controls which caused severe edema in interstitial connective tissue, especially in HDT group (P<0.05). The mean percentage of seminiferous tubules with germinal cells dissociation and cell depletion ratio in HDT and LDT groups were 29.84 ± 14.17, 14.72 ± 5.52 respectively.

Seminiferous tubule diameter in both of treated groups decreased significantly in comparison with the control group (P<0.05). Also, seminiferous tubule diameter showed a significant difference between LTD group and HDT group (P<0.05) (Figure 3). The mean seminiferous tubule diameter in the control, LTD, and HDT group were 292.73±1.64μm, 255.34±1.95 μm, and 247.85±2.72 μm, respectively. Seminiferous epithelial thickness in both of treated groups decreased significantly in comparison with the control group (P<0.05). No significant difference in epithelial thickness was found between LTD and HDT groups (P>0.05).
The mean seminiferous epithelial Thickness in the control, LDT, and HDT groups were 80.16±0.63 µm, 77.28±0.71 µm, and 74.82±0.89 µm, respectively (Figure 4).

![Tubule Diameter graph]

**Figure 3** - The effect of buserelin on seminiferous tubule diameter in adult rats. Significant differences were seen in the low dose and high dose groups in comparison with the control group *(P=0.000). There was also a significant difference between the low dose and high dose groups** *(P=0.034).

![Epithelial Height graph]

**Figure 4** - The effect of buserelin on seminiferous epithelial Thickness (height) in adult rats. Significant differences were seen in the low dose and high dose groups in comparison with the control group *(P=0.033) *(P=0.000) respectively.

**TUNEL results**

The percentage of TUNEL-positive cells was defined by counting them in the same number of seminiferous tubules (Figure 5). TUNEL-positive cells in testicular tissues of treated groups were likened with controls. The HDT rats showed a significantly increased percentage of apoptotic cells in seminiferous tubules in comparison with the controls (p<0.05). There was no significant difference between HDT or control groups compared with the low dose group (P>0.05). The mean percentage of apoptotic cells in seminiferous tubules of LDT, HDT, and control groups were 4.58±0.78%, 5.75±0.51%, 2.19±0.19%, respectively (Figure 6).

![TUNEL results images]

**Figure 5** - Percentage of apoptotic cells in seminiferous tubules. (a) Dense dark TUNEL-positive cells were found in the control group normally. (b) Dense dark TUNEL-positive cells increased but not significantly in low dose treated group. (c) Dense dark TUNEL-positive cells increased significantly in high dose treated group. (Magnification: x400).

![Percentage of Apoptotic cells graph]

**Figure 6** - The effect of buserelin on apoptosis of seminiferous epithelial cells in adult rats. Significant differences were observed between high dose and control groups *(P=0.007).
Discussion

Buserelin as a GnRH agonist is a part of approved therapeutics for the treatment of male infertility, prostate cancer and for males with delayed puberty. GnRH agonists act in a similar way of natural GnRH. They trigger an initial gonadotropins secretion which causes a subsequent testosterone production, but after such initial stimulation, they exert a long-term inhibitory effect via receptors. It is reported that the use of buserelin significantly disrupts endocrine function and spermatogenesis in the testes. In keeping with the earlier study on the impact of buserelin related testicular changes immature Wistar male rats, the present study was sought to assess the potential damage of buserelin as a GnRH agonist in adult Wistar rats. Inhibitory activity in the gonads and reproductive performance reduction occur by the increase of the binding affinity of GnRH agonists to their receptors and through occupying the site of these receptors.

This study show that the effects of buserelin on the reproductive system are a dose dependent impact and increasing of dose from 300 to 500 µg/kg reduced the serum testosterone levels none significantly. LH and probably FSH are essential for the normal operation of Leydig cells. Therefore, reducing the gonadotropins as a result of pituitary desensitization in response to the GnRH agonist can be a justification for the inhibitory effect of the daily use of GnRH agonist. The dysfunction of Leydig cells through down-regulation of LH receptors and daily use of GnRH agonist is another factor in decreasing blood levels of testosterone. GnRH agonist has an inhibitory effect on spermatogenesis due to decreasing testosterone level. There is another study which pointed to the effect of GnRH agonist in the treatment of prostate cancer so that reduced testosterone levels by GnRH agonist can be effective in reduction the number of cancer cells.

The dose-dependent effect of buserelin is evidently observed in morphometric results. Buserelin significantly reduced the diameter of seminiferous tubules in a month after the first injection. In the control group, normal spermatogenesis was maintained as a result of interaction between sertoli cells and germ cells. Testicular damage begins after receiving the low dose of buserelin (300µg/kg), and some seminiferous tubules with damaged germ cells exist but a higher numbers of healthy tubules are evident as well. High dose (500µg/kg) of buserelin caused more severe changes as the tubules with damaged epithelium and less number of healthy tubules. Apoptotic cell death in HDT group was significantly higher than the other groups and accumulations of buserelin induced apoptotic cells produces pathologic changes in seminiferous tubules diameter and epithelium Thickness which reported by others as well. Steroid hormones such as testosterone and FSH play an important role in the control of physiological cell death (apoptosis). Hence, GnRH agonist induces a decreased level of gonadotropins which leads to reduced level of testosterone, exacerbate destruction of seminiferous tubules germ cells and causes a significant increase in the number of destroyed germ cells with morphological changes.

In this study, the apoptotic nuclei were observed clearly in spermatogonia and spermatocytes which express GnRH receptors and are targets of GnRH agonist. It is reported that the possibility of stopping the process of spermatogenesis is higher in spermatogonia and spermatocytes. The current findings are in agreement with the study conducted by Aiudi et al. on dogs.

Conclusion

Buserelin induces apoptotic cell death in spermatozoa lineage and decreases epithelium Thickness of seminiferous tubules in the adult rat testes in addition to disruption cell association in seminiferous tubules.
References


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