Ghrelin modulates testicular germ cells apoptosis and proliferation in adult normal rats

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Under normal condition in the most mammals, spermatogenesis is closely associated with the balance between germ cells proliferation and apoptosis. The present study was designed to determine the effects of ghrelin treatment on in vivo quality and quantity expression of apoptosis and proliferation specific indices in rat testicular germ cells. Twenty eight adult normal rats were subdivided into equal control and treatment groups. Treatment group received 3 nmol of ghrelin as subcutaneous injection for 30 consecutive days or vehicle to the control animals. The rats from each group (n = 7) were killed on days 10 and 30 and their testes were taken for immunocytochemical evaluation and caspase-3 assay. Immunohistochemical analysis indicated that the accumulations of Bax and PCNA peptides are generally more prominent in spermatocytes and spermatogonia of both groups. Likewise, the mean percentage of immunoreactive spermatogonia against Bax increased (P < 0.01) in the ghrelin-treated group on day 10, while despite of 30% increment in the Bax level of spermatocytes in the treated rats on day 30, however, it was not statistically significant. During the experimental period, only a few spermatogonia represented Bax expression and the changes of Bax immunolabling cells were negligible upon ghrelin treatment. Likewise, there were immunostaining cells against Bcl-2 in each germ cell neither in the control nor in the treated animals. In fact, ghrelin balanced Bax/Bcl-2 ratio toward at increase of Bax level in the spermatocytes and therefore may stimulate apoptosis in these germ cells. In contrast, ghrelin administration significantly suppressed proliferation-associated peptide PCNA in the spermatocytes as well as spermatogonia (P < 0.05). Whereas, caspase-3 activity did not show any marked alteration during the experiment in both groups (P > 0.05). Upstream of Bax substance parallel to down-regulation of PCNA demonstrate that ghrelin may prevent massive accumulation of germ cells during normal spermatogenesis. These observations also indicate that ghrelin may be considered as a modulator of spermatogenesis in normal adult rats and could be potentially implicated for abnormal spermatogenesis in some testicular germ cell tumors.

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1. Introduction

Onset of spermatogenesis is associated with an apoptosis wave that limits its efficiency during the first cycles in the most mammals [1]. The seminiferous epithilium is a highly proliferating tissue in which germ cell degeneration is a constant feature [2]. During normal spermatogenesis, more than half of the germ cells undergo apoptosis [3].

Expression of ghrelin has been demonstrated in mature Leydig cells of rat and human. In addition, expression of the functional ghrelin receptor, GHS-R1a, has been shown in both Sertoli and Leydig cells. On the other hand, expression of GHS-R1a in the seminiferous tubules strongly suggests that the seminiferous epithilium might be a target for ghrelin action and directly regulates seminiferous tubules function [4]. Thus, it is expectable that ghrelin may control the key gonadal functions, proliferation and apoptosis, in the rat testis.

Recently, numerous studies have documented the direct action of ghrelin in the modulation of apoptosis in different cell types. For example, it has been reported that ghrelin inhibits apoptosis in several cells such as cardiomyocytes [5], pancreatic β cells [6] and pituitary lactotrophs [7], but promotes it in chick ovarian granulosa cells [8], endothelial cells [9], aldostroma and adencarcinoma derived cells [10]. However, the role of ghrelin in the testicular germ cells apoptosis and proliferation has not been yet studied. Therefore, the present work attempted to explore the possible involvement of ghrelin treatment on the apoptosis and proliferation indices in the rat testicular tissue.
2. Materials and methods

2.1. Reagent and drugs

Expressions of Bax, Bcl-2 and PCNA were detected using available standard immunohistochemical kits provided from Dako Company (Glostrup, Denmark). Caspase-3 activity was determined using commercial colorimetric kit obtained from Biovision Research Products (CA9403, Mountain View, USA). Rat lyophilised acylated ghrelin (n-octanoylated research grade) were purchased from Tocris Cookson Ltd. (Bristol, UK).

2.2. Animals

Twenty eight sexually mature male Wistar rats with 70 days of age obtained from Razi Research Institute (Karaj, Iran) were applied for this study. The animals were maintained under constant conditions of photoperiod (12 light:12 darkness) in an animal pplied for this study. The animals were maintained under constant conditions of photoperiod (12 light:12 darkness) in an animal room in groups of seven rats per cage and controlled temperature (21–24 °C). All investigations were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals. All animals were treated humanely and in compliance with the recommendations of Animal Care Committee for the Lorestan University of Medical Sciences (Khorramabad, Iran).

2.3. Experimental design

The rats were randomly assigned to either control or treatment groups (n = 7 in each group). To monitor the effects of ghrelin on germ cells apoptosis and proliferation, a general protocol of subcutaneous injection of ghrelin (3 nmol/100 μl saline) or 100 μl vehicle (physiological saline) to the control group was applied once a day for 30 consecutive days. This dose of ghrelin used in our in vivo setting, is close to that induced by fasting. Because, exogenous administration of 1 nmol of ghrelin is able to induce a significant elevation (2.4–2.6-fold increase) in serum levels of total ghrelin 1 h after injection [11], whose magnitude is in the range of that induced by fasting [12]. The rats from each group (n = 7) were killed upon diethyl ether anesthesia by decapitation on 10th and 30th days of treatment.

2.4. Sampling and tissue preparation

Immediately after rat killing, both testes were removed and carefully cleaned of fat and adhering. One testis from each group stored at –80 °C for later caspase-3 assay. Preparation of another testis in both groups was made prior to immunocytochemical analysis of apoptosis and proliferation. In short, the samples were fixed in Bouin’s solution and following dehydration in a descending series of ethyl alcohol, were cleared in xylene and embedded in paraffin.

2.5. Determination of caspase-3 activity

The caspase-3 activity was measured using a caspase-3 colorimetric assay kit according to the manufacturer’s instructions by a spectrophotometer (Jenway, UV/Vis, Essex, UK). The caspase-3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase-3, resulting in the release of the p-nitroaniline (pNA) moiety. p-Nitroaniline has a high absorbance at 405 nm (εM = 10.5). The concentration of the pNA released from the substrate is calculated from the absorbance values at 405 nm. Enzyme activity was defined as produced micromole pNA/min/milligram tissue protein, and expressed as U/mg protein.

2.6. Bax, Bcl-2 and PCNA immunostaining

Immunocytochemical analysis was carried out to identify Bax, Bcl-2 and PCNA expressions in the testicular germ cells. 3 μm de-waxed and rehydrated sections were immersed in target retrieval solution (pH 9.0) and boiled water bath for 20 min at 98 °C to delivered masked antigens. The sections were then dipped in 3% H2O2 for 10 min to block endogenous peroxidase and nonspecific background staining was blocked by incubating the sections for 3 min in 1% normal rabbit serum. The slides were incubated with the following antibodies: polyclonal rabbit antibody against Bax protein (dilution 1:40; Dako Corporation Carpinteria, CA, USA), mouse monoclonal anti-PCNA (Clone PC10; dilution1:100; Dako, Glostrup, Denmark) and monoclonal antibody against Bcl-2 (Clone124; dilution 1:50; Dako, Glostrup, Denmark) followed by rinsing in TBS. Thereafter, all sections incubated with biotinylated secondary antibody (Envision kit: Dako, Glostrup, Denmark) at room temperature for 30 min. The preparations were incubated with liquid diaminobenzidine tetrahydrochloride substrate (DAB, ready to use) for 10 min and were counterstained with Mayer’s hematoxylin, dehydrated, and mounted in Diatex at the end of processing. Negative control slides in the absence of primary antibody were also included [13].

2.7. Statistical analysis

Results were analyzed using SPSS/PC program version 16 (SPSS Inc., Chicago, IL, USA). All data were tested for normality followed by homogeneity of variances by Levene static test. When the variances were homogenous, the activity of caspase-3 and the percentage of cells immunostained against Bax and PCNA but not Bcl-2 (by counting of at least 100 cells per chamber slide) either in the control or in the treated animals were compared between groups on days 10 and 30 separately by independent sample t-test. Values were considered to be statistically significant at P < 0.05.

3. Results

Apoptosis-regulating proteins Bax and Bcl-2 as well as proliferation-associated peptide PCNA were identified immunocytochemically in seminiferous germ cells (Figs. 1–3). Generally, the occurrence of Bax expression was much pronounced in the spermatocytes compared to the spermatogonia either in the control or in the ghrelin-treated groups, with no reaction in Sertoli cells (Fig. 1). Notably, ghrelin treatment significantly increased the population of immunoreactive cells against Bax in the spermatocytes on day 10 (P < 0.01). Although, 1.3-fold increment was also seen in the Bax positive cells in these cells by day 30 (12.90 ± 3.18% versus 16.83 ± 3.17% in the control and treated rats, respectively), however, they did not differ statistically (P > 0.05).

On the other hand, ghrelin injection did not change the proportion of spermatogonia labeling for Bax neither on day 10 nor at day 30 (P > 0.05), so that the alterations of Bax immunostained cells were negligible upon ghrelin exposure (Fig. 4). It seems that the hormone could not significantly affect the Bax expression in the cytoplasmic region of these cells. Furthermore, there were not any immunoreactivity against Bcl-2 protein in seminiferous germ cells of both groups and long term administration of ghrelin was not able to modify the expression of Bcl-2 in the rat testis (Fig. 2).

Immunocytochemical analysis revealed that the brown intensive nuclear stained cells against PCNA were mainly localized in the spermatocytes and with a lesser extent in the spermatogonia (Fig. 3). Interestingly, administration of ghrelin suppressed the
number of immunostained spermatocytes for PCNA in both experimental days \((P < 0.05)\). Such a similar finding was also obtained for spermatogonia by day 10 \((P < 0.05)\). The proportions of positive cells against PCNA antigen in the germ cells are presented in Fig. 4.

Attempt for caspase-3 assay in our experiment showed that no considerable changes were occurred in the enzyme activity following ghrelin treatment. So that, the mean activity of enzyme ranged between \(2.67 \pm 0.60\) and \(1.67 \pm 0.33\) U/mg protein on day 10 or \(2.00 \pm 0.30\) and \(2.19 \pm 0.45\) U/mg protein on day 30 in the control and ghrelin-exposed animals, respectively (Fig. 4).

4. Discussion

To our understanding, this is the first study showing modulatory effects of ghrelin on testicular germ cells apoptosis and proliferation under normal condition. These actions are mainly through up-regulation of Bax protein expression consistent with down-regulation of PCNA level, however, are not associated with caspase-3 activity and also not depend on the modifying of Bcl-2 expression.

Sperm production depends on the balance between spermatogenesis and apoptosis. There are numerous \((9–11)\) cell divisions of spermatogonia and 2 divisions of spermatocytes that build the germ cell population, thus the excess of these germ cells undergo apoptosis. Loss of germ cells may also occur under pathological status such as deficiency of gonadotropins or androgens [14]. Apoptosis was shown to play a major role in onset and maintenance of spermatogenesis. This will lead to maintenance of germ cell to Sertoli cell ratio and allowing spermatogenesis to go on [1]. Thus, the induction of apoptosis depends not only on the balance between pro- and anti-apoptotic members of the Bcl-2 family, but also on their intracellular localization [15]. An important regulatory step in apoptosis occurs at mitochondrial membranes where members of Bcl-2 family of proteins either promote (Bax, Bad and Bak) or prevent (Bcl-2, Bcl-w and Bcl-xl) membrane permeability [16]. Among germ cells, spermatogonia and spermatocytes are the main targets of apoptosis both in physiological or extra-physiological conditions [1].

Immunohistochemically, Bax is mainly localized in spermatogonia and spermatocytes, but not in spermatids in the rat normal testis. Bax has been also detected in Sertoli cells, but at much lower
levels than in spermatogonia and spermatocytes [17]. The results obtained in the current study showed that the expression of Bax in spermatocytes were more prominent than those observed in the spermatogonia. The surprising finding was that ghrelin increased the mean population of germ cells stained against Bax substance, in which the percentage of Bax positive germ cells was significantly higher in the spermatocytes on day 10. Although low labeling for Bax was identified in the spermatogonia in both control and ghrelin-exposed testes, however, the changes were not statistically significant. Similar finding of low Bax expression in the spermatogonia was also observed in our laboratory in the rat testis with heat-induced apoptosis and also when treated by ghrelin (unpublished data). These results demonstrate that ghrelin may up-regulate the expression of pro-apoptotic factor Bax particularly in spermatocytes and, therefore the lower population of germ cells could reach to the later stages of spermatogenesis cycle.

It should be considered that the interaction among Bcl-2 family members is necessary to maintain cell equilibrium in highly proliferating tissues. For example, ghrelin influence the mitochondrial pathways of apoptosis and it showed increased Bcl-2 protein expression and decreased Bax protein expression in cardiomyocytes [5], pituitary lactotrophs [7] and pancreatic β cells [6]. Therefore, we designed to determine the Bcl-2 content immuno-histochemically in the testicular germ cells in order to explore the probable role of ghrelin modulatory effects through changes of Bcl-2 expression in the testis. However, no significant alterations were observed in the number of Bcl-2-positive germ cells, and ghrelin was not capable to enhance Bcl-2 reactive cells in all of the experimental groups. This is in agreement with previous reports showing that Bcl-2 is absent in the mature rat testis [17,18]. Bcl-xL level, a homolog of Bcl-2, is also relatively low in the adult rat testes [17] and it is localized merely in round spermatids [19], which express neither Bcl-w nor Bax nor Bak.

Higher expression of Bax and undetectable level of Bcl-2 in spermatogonia and particularly in spermatocytes may suggest that these type of cells are more susceptible to apoptosis in normal testis and also show that ghrelin apoptotic effects is mediated through increase in Bax to Bcl-2 ratio, which is the critical determinant of cell fate. However, it is remained unclear whether other members of Bcl-2 subgroups such as Bcl-w, Mcl-1 or A1/Bfl-1 may have a possible role in the ghrelin apoptotic modulatory effects in the testis or not, which should be investigated in future studies.

There are some mechanisms that can be attributed for ghrelin apoptotic controlling function in the rat testis. The pattern of ghrelin and its functional receptor (GHS-R1a) expression in the testis are species-specific. In the rat, ghrelin immunoreactivity has been localized in interstitial Leydig cells, and GHS-R1a has been presented mainly in Sertoli and Leydig cells as well as primary and secondary spermatocytes [20]. Thus, ghrelin may participate in the regulation of testicular function. On the other hand, involvement of testosterone in regulating programmed cell death, beside cell proliferation and differentiation during spermatogenesis is well documented [2,17]. Likewise, it has been widely demonstrated that withdrawal of gonadotropins and/or testosterone increases testicular germ cell degeneration and it appears that apoptosis of germ cells in the testis is under the control of FSH and testosterone [2,17,21–23]. Notably, recent evidences indicate that ghrelin is able to inhibit stimulated testicular testosterone secretion in vitro [4,11,24] and also chronic-low infusion of ghrelin to male rats resulted in significant decrease in circulating LH and FSH concentrations [25]. More recently, Wang et al. [20] proved that administration of 1 and 3 nmol of ghrelin could significantly inhibit testosterone secretion in vivo and also the expression of androgen receptor mRNA was shown to reduce with 3 nmol of ghrelin in that study. Therefore, it may be concluded that ghrelin probably exerts its modulatory functions in the rat testis indirectly through inhibition of testosterone secretion or via decrease in FSH circulating level. The possible impact of these regulatory actions results in upstream of cytoplasmic expression of Bax as well as down-regulation of nuclear proliferation-associated peptide PCNA both in spermatogonia and spermatocytes. The findings obtained in the present work are relatively in consistent with our previous studies in the rat testis [26] and ovary [27], where we investigated...
the role of ghrelin on the morphometry and intracellular changes in rat testicular and ovarian tissues using stereology and electron microscopy techniques and similarly, some signs of apoptotic events and anti-proliferative effects were observed in those investigations.

Russel et al. [28] indicated that Bax is required for normal spermatogenesis in mice. Despite of its pro-apoptotic function, previous results found that Bax-deficient mature male mice demonstrate increased cell death and dramatic testicular atrophy [29]. The excess germ cells present in Bax-deficient animals is substantial and may overwhelm the Sertoli cell’s ability to support such a massive increased cell. This finding represents the novel function of Bax in spermatogenesis and explains that preventing the pro-apoptotic activity of Bax is sufficient to disrupt normal spermatogenesis. They also suggest that lack of Bax results in overproduction of spermatogonia, hyperplasia of the seminiferous tubules, and a block in normal maturation of spermatocytes [28].

It is well known that caspase-3 is an essential effector molecule for carrying out programmed cell death in eukaryotic cells [30] and is activated in the final stages of cellular death [31]. The Bcl-2 family has a controlling role in the apoptotic signal, whereas caspases play a central role in cell protein degradation [32]. Ghrelin have been shown to reduce caspase-3 activation in ovaries of pig [31] and chicks [8], as well as human endothelial cells [33]. However, chronic exposure to ghrelin failed to change caspase-3 activity in our in vivo setting. It seems that apoptotic modulatory effect of ghrelin in the rat testis is not associated with caspase-3 activation. Likely, a prolonged treatment by ghrelin or higher doses may be needed to induce any change in caspase-3 activity in the testis. Certainly, further researches are necessary to clarify this hypothesis.

Immunocytochemical localization of proliferating cell nuclear antigen, PCNA, revealed that ghrelin has significantly suppressed germ cells proliferation. Down-regulation of PCNA level in spermatocytes and spermatogonia may prevent excess germ cells

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**Fig. 4.** Increase in the percentage of spermatocytes (A) containing Bax substance upon treatment. Much lesser number of spermatogonia (B) showed Bax protein with no significant change in its expression in ghrelin-exposed animals. (C, D): Suppression of PCNA positive cells is seen in the spermatocytes (C) and spermatogonia (D) by ghrelin administration. (E): The mean activity of caspase-3 in the control and treated animals. Data did not show significant changes between experimental groups (P > 0.05). Bars represent mean ± SEM. Comparison between groups was made using independent sample t-test. All means marked with * (P < 0.05) and ** (P < 0.01) are significantly different from the control. NS: not significant.
References

14. M. Parvinen, J. Toppari, Function of stem cell factor as a survival factor of spermatogonia and localization of messenger ribonucleic acid in the rat seminiferous epithelium [36]. This is in parallel to significant decline in PCNA level in germ cells in the ghrelin-treated animals in the current study.
15. In conclusion, the results of the present investigation demonstrate the novel modulatory effects of ghrelin in the germ cells of rat normal testis, which is mainly mediated through up-regulation of Bax expression and suppression of PCNA level. The analysis of the factors involving in testicular apoptosis and proliferation and their controlling mechanisms may have potential important implications for better understanding of impaired spermatogenesis and the genesis of some germ cell tumors.

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