Effects of Ghrelin on Testicular Ischemia/Reperfusion-Induced Injury

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Abstract- Ischemia-reperfusion injury is a possible cause of testicular damage and infertility after testicular torsion and detorsion. The purpose of this study was to evaluate the effect of ghrelin on testicular Ischemia-reperfusion damage. A total of 30 adult male rats were selected for the study and divided randomly into 3 groups, each containing 10 rats. Animals in the testicular torsion and ghrelin treated groups were subjected to unilateral 720°counterclockwise testicular torsion for 1 hour, and then reperfusion was allowed after detorsion for 7 and 30 days. The ghrelin-treated group and the other two groups received intraperitoneally 40 nmol of ghrelin and physiological saline 15min before detorsion, respectively. The animals were sacrificed at the end of reperfusion times, and their testes were taken for later histopathological examination. The seminiferous tubules diameter, germinal epithelium height, as well as volume densities in testicular torsion / detorsion plus saline group, were significantly lesser versus control group, which clearly indicates an ischemia-reperfusion injury. Ghrelin treatment resulted in a partial increment in examined histological parameters on day 30 after reperfusion. Current results showed that ghrelin ameliorates the testicular ischemia-reperfusion damage.

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Introduction

Testicular torsion is one of the emergency conditions which require immediate surgery to reperfuse the affected testis, however, attempt to reperfuse of ischemic tissue may cause further damage to the testis. Many studies reported a loss of germ cells and disruption of the seminiferous epithelium after ischemia-reperfusion (IR) injury of the testis (1,2). A possible cause of IR damage is the reactive oxygen species (ROS) produced during its process (3). This ROS interact with lipids, proteins and nucleic acids leading to the loss of membrane integrity, structural or functional changes in proteins and genetic mutations, respectively (4). In a tissue like the testis, with its high rates of metabolism and cell replication, excessive production of ROS can especially be damaging, which makes the antioxidant capacity of the tissue very important. Prevention of reperfusion injury using a combination of enzymes and drugs has been studied along with the assessment of histopathological changes after testicular torsion/detorsion (2,3,5). They were intended to inhibit oxidative stress. For instance zinc aspartate, curcumin and dexamethasone reduce IR injury and increase the activity of antioxidant enzyme systems (6-8).

Ghrelin is a 28 amino acids orexigenic hormone produced principally in the stomach and has been identified as the endogenous ligand for the growth hormone secretagogue receptor (GHSR) (9). Ghrelin and its receptors were detected in testicular tissues indicating that the peptide may play a role in testicular regulation (10). The possible involvement of ghrelin in the protection of numerous tissues against IR injury has been shown (11). Antioxidant properties of ghrelin have been demonstrated in the rat testis (12).

Recently, we showed that administration of ghrelin increases antioxidant enzyme activities and reduces the lipid peroxidation in the testicular tissue exposed to IR (12).

In spite of remarkable antioxidative properties of ghrelin, few studies have been conducted on IR injury using ghrelin. Therefore, the present study was designed to investigate the histopathological effect of ghrelin on the testes of Wister rats following an IR injury. The

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results of this study may provide therapeutically active compounds for the treatment of testicular IR injury.

Materials and Methods

Animals

Male Wistar rats weighing 220 to 250 g were housed in a temperature- controlled room (24 ± 1°C) on 12 h light–12 h dark cycle with free access to food and water. Animals were treated humanely and all procedures complied with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory animals. The Medical Laboratory Animal Management Committee of Lorestan University of Medical Sciences approved all experiments.

Drugs

Rat lyophilized acylated ghrelin (M.W.=3314.83) were purchased from Tocris Cookson Ltd. (Bristol, UK) and a solution of Ghrelin was prepared by normal saline (40 nmol/1 ml).

Experimental groups

In total, 30 animals were selected for the study and divided randomly into 3 groups, each containing 10 rats. Group 1 was the control group (CS), and animals in this group underwent sham operation without the application of the torsion and received intraperitoneally 1 ml normal saline. Group 2, the torsion/detorsion plus normal saline (T/D-S) group which underwent 1 hours of testicular torsion followed by 7 and 30 days of detorsion. The animals in this group received saline intraperitonally15 min before detorsion. Group 3, ghrelin treated group (T/D-G) was the test group which received ghrelin (40 nmol) 15 min before the testicular detorsion. The dose of the drug was chosen from the reports of the pilot study conducted in our laboratory. In group 3, torsion and detorsion times was the same as group 2. In all groups, five rats from each group after inhalational anesthesia by diethyl ether (May & Baker Ltd., Dagenham, England) in a jar, were sacrificed by decapitation at 7 and 30 days after detorsion and unilateral orchiectomy was then performed, and the testis was processed for histopathological examination.

Surgical procedure

Surgical torsion was carried out as described by Turner et al. modifications (13). The animals were generally anesthetized with intraperitoneal injection of ketamine HCl (75 mg/kg) and xylazine (8 mg/kg) and the surgical operation described below was performed. The skin of scrotal area was shaved and then prepared with 10 % povidone iodine solution. A right-sided mild scrotal vertical incision was performed for access to testis. Torsion was created by twisting the right testes 720° in a counter-clockwise direction and maintained by fixing the testes to scrotum with a 4–0 silk suture passing through the tunica albuginea and dartos. After 1 hour of ischemia, the suture was removed, and right testes were detorted and replaced into scrotum for 7 and 30 days of reperfusion. During sham operation, the right testes were brought through the incision and then replaced without twisting, and a silk suture was placed through the tunica albuginea. After each surgical intervention, the incision was closed using silk sutures.

Histopathological evaluation

Samples were fixed in Bouin's solution, and the following dehydration in a descending series of ethyl alcohol were cleared in xylene and embedded in paraffin. Paraffin sections of testes were cut at 5 μmon a rotary microtome, mounted on slides and stained with hematoxylin-eosin (H&E) and examined under a light microscope (Olympus CX-31, Philippines). The tubular diameter, height of seminiferous tubules epithelium was measured using an ocular micrometer. At least 10 tubular profiles that were round or nearly round were chosen randomly and measured for each animal per group. The epithelial height was obtained in the same tubules utilized to determine tubular diameter. The volume densities of all testicular tissues were determined by light microscopy, using a 441-intersection grid placed under the ocular of the light microscope. Ten randomly chosen fields (4410 points) were scored for each animal at 100 X power field. The points were classified as seminiferous tubules (epithelium and lumen); and interstitial stroma (14).

Statistical analysis

One-way ANOVA was used for statistical analysis of data among all groups. Multiple comparisons were made using Tukey posthoc test with $P< 0.05$ considered statistically significant. Data are expressed as the mean ± SEM.

Results

Examination of the H&E stained sections of experimental groups is depicted in Figure 1. Histopathological evaluation of testes of the control group as shown in Figure 1 revealed multiple rounded seminiferous tubules with regular outlines. They were

lined by multiple layers of germinal epithelium at different stages of spermatogenesis. On the other hand, examination of the sections of testes in both saline and ghrelin treated T/D groups on day 7, showed diminished tubular diameter with wave-like hyaline thickening of basement membrane, decreased the thickness of the germinal epithelium, associated with intratubular giant cell formation and vacuolization of cytoplasm. Spermatogenesis was arrested in nearly all tubular structures which principally were lined by Sertoli cells and highly reduced numbers of spermatogonia with no evidence of spermatocytes. Interstitial stroma was expanded by edema that infiltrated with mononuclear cells. These results indicated that the surgical procedure was effective in inducing the required level of testicular damage. However, histological observation of testicular sections on day 30 after reperfusion displayed no recovery of tubular structures in a saline group, the multinucleated giant cells formation, cytoplasm vacuolation principally in spermatogonia and Sertoli cells and high population of inflammatory cells were striking but only a few to moderate spermatocytes reappeared, whereas fairly well-differentiated and more proliferated of spermatocytes with lack of spermatids were revealed in the most tubules, in ghrelin treated group. Figure 2 depicts the mean of tubular diameter as well as germinal epithelium thickness at different experimental days in each group. The mean values of these two parameters in T/D-S and T/D-G groups showed the significant difference as compared with the control group on both 7 and 30 days after reperfusion ($P<0.001$). A significant increase in these parameters was evident in the T/D-G group only on 30 after reperfusion, and ghrelin could promote tubular recovery by this time when compared with T/D-S group ($P<0.05$). Whereas the mean diameter of tubules and germinal epithelium height showed greater values in T/D-G group compared to the T/D-S animals ($P<0.05$), these parameters remained lesser than the control rats.

The percentages of volume densities of different testicular compartments are summarized in Fig 3. Concerning intertubular space and tubular lumen, significant increase in the volume densities of these parameters was observed in both T/D-G and T/D-S groups on days 7 and 30 after reperfusion compared to control group but simultaneously, a significant decrease in the volume density of seminiferous epithelium was found at the both period of reperfusion. Administration of ghrelin markedly reversed the volume densities of tubular lumen and seminiferous epithelium to their normal values when compared with T/D-S group. In contrast, no significant change for intertubular space was evidenced at any period of reperfusion in ghrelin treated group compared to T/D-S animals.

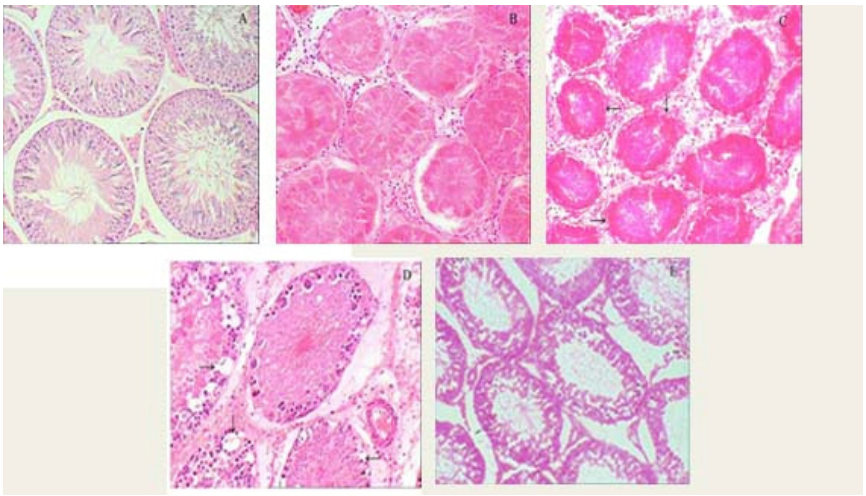

Figure 1. Photomicrographs of testicles in the control (A) and torsion/detorsion groups (B-E). A: well-developed seminiferous tubules with normal structures. (B, C): Massive degeneration of germinal epithelium, sclerosis of hyalinized seminiferous tubules with broad intense interstitial edema as well as invasion of inflammatory cells and wavy-like thickened hyaline membrane (arrows) on day 7 after reperfusion following saline (B) and Ghrelin (C), multinucleated giant cells formation, cytoplasmic vacuolation (arrows) with increasing numbers of few spermatocytes; Saline on day 30 (D). Relative well-formed seminiferous tubules are in contrast to the saline groups. However, the spermatogenesis is not developed, but giant cells and vacuolation are not produced, and interstitial edema is in lower content; Ghrelin on day 30 (E). (A-E); HE (100×)

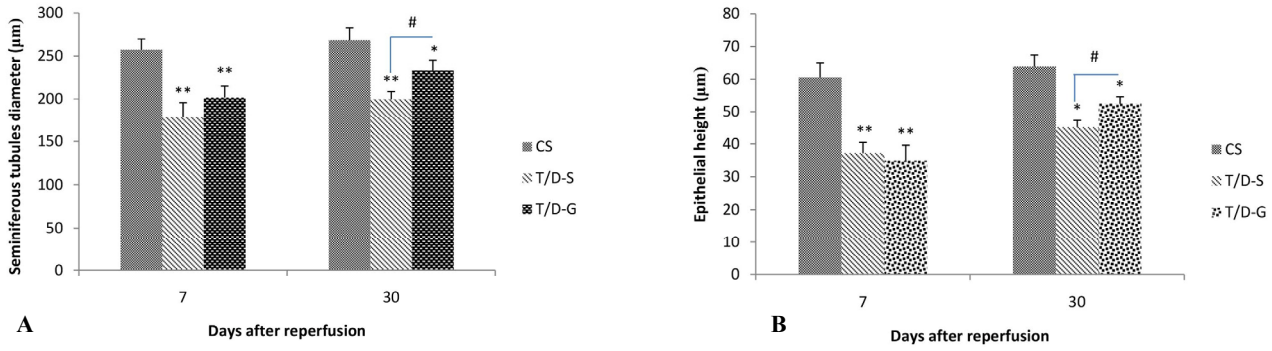

Figure 2. The means of tubular diameter (A), germinal epithelium thickness (B) in all groups at each experimental days. Means marked with * (P<0.05), ** (P<0.001) are significantly different from control and means marked with = (P < 0.05) shows significantly difference between T/D-S and T/D-G groups. Groups were defined as: CS, control-saline; T/D-S, portion/detortion-saline; T/D-G, portion/detortion-ghrelin. Values are expressed as mean ± SEM.

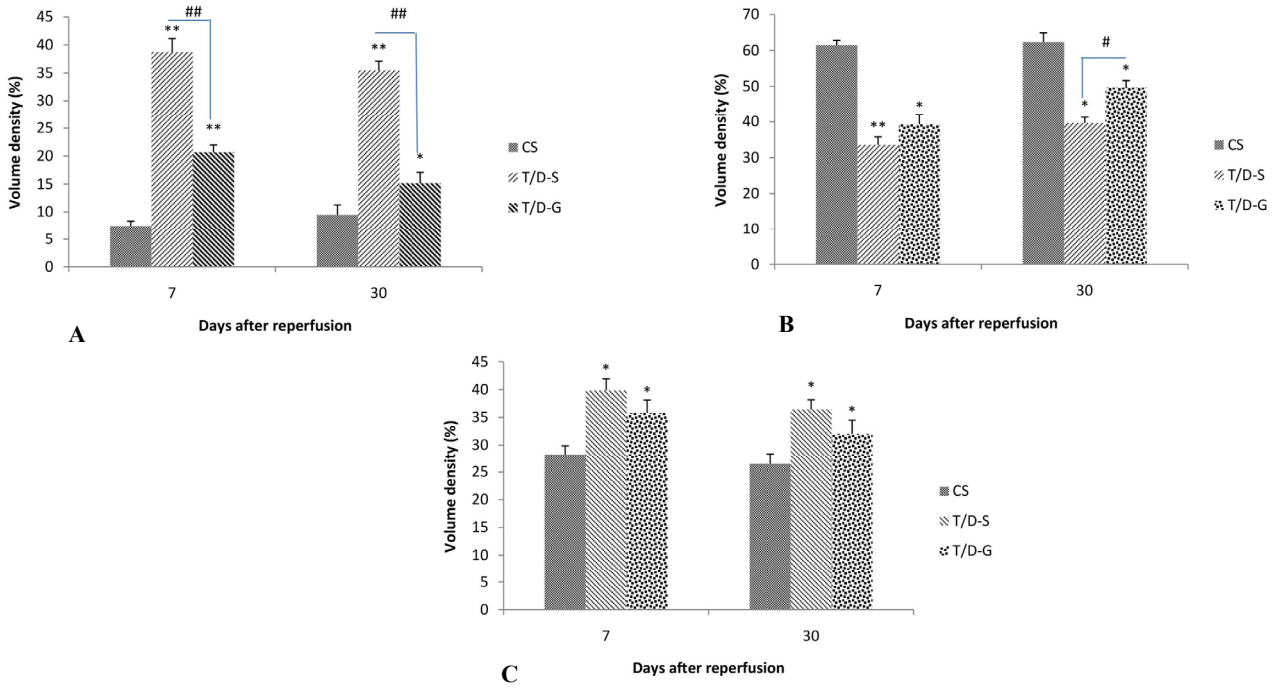

Figure 3. Percentages of volume density of main testicular components: tubular lumen (A), germinal epithelium (B) and interstitial space (C), calculated at 7 and 30 days after reperfusion. All means marked with * (P<0.05), ** (P<0.001) are significantly different from the control (CS) in each day and means marked with = (P < 0.05), == (P<0.001) shows significantly difference between T/D-S and T/D-G groups. Values are expressed as mean ± SEM.

Discussion

In the current study, untreated rats that were subjected to ischemia (torsion) for 1 hour followed by reperfusion (detorsion) for 7 and/or 30 days showed a testicular injury with apparent seminiferous tubular necrosis. Present results also show that the seminiferous tubular diameter and thickness of germinal epithelium

decreased significantly in these untreated rats when compared to the control group on days 7 and 30 after reperfusion. Administration of ghrelin prior to detorsion significantly increased seminiferous tubular diameter and thickness of germinal epithelium and attenuated testicular tissue damage only in the group with 30 days reperfusion period. It has been suggested that a thicker germinal epithelium in IR injury could be the sign of an effective treatment (15). Hence, these results indicated that IR-induced testicular injury may be successfully treated with the ghrelin. In our knowledge, this study is the first to show the protective effects of ghrelin on testicular ischemia/reperfusion-induced degeneration in the rat.

There are reports about adverse effects of post-ischemia reperfusion on testicular function, including germ cell loss and disruption of the seminiferous epithelium (3,16). It is widely accepted that reactive oxygen species (ROS), such as hydroxyl radicals, superoxide anions, singlet oxygen and hydrogen peroxide (H2O2) has a significant role in the testicular ischemia-reperfusion injury (14). Under normal conditions, free radicals are produced, and their effects are counterbalanced by way of their own antioxidant mechanisms, including enzymatic and non-enzymatic antioxidant systems (17). When ROS generation exceeds the defense mechanism's capacity to control, a condition referred to as oxidative stress exists and contributes to reversible or irreversible cell injury (6,18). The first line of cellular defense against oxidative stress is the family of superoxide dismutases, glutathione peroxidases (GPXs), and catalase enzymes. They are the main free radical-scavenging enzymes which decompose superoxide radicals and H2O2. ROS is overproduced by two pathways in post-ischemic tissues. Ischemia causes an increase in intracellular hypoxanthine as a result of ATP breakdown and then, during reperfusion, xanthine oxidase converts hypoxanthine to uric acid plus large quantities of superoxide radicals in the presence of oxygen (19). Neutrophil recruitment is the other source of ROS production (1). The increase in proinflammatory cytokines (TNF-α, IL-1β, and IL-8) after IR enhances the capture and attachment of blood neutrophils to the vascular endothelium (20). Neutrophils transmigrate through the endothelium into the interstitium of the testis and then release factors such as ROS.

Oxidative damage can occur to many classes of molecules particularly lipids in a state called lipid peroxidation (21). It is widely accepted that lipid peroxidation is a mechanism of cellular injury (22). The lipid peroxidation, indicating the presence of enhanced ROS due to I/R injury has been shown to increase after testicular torsion and detorsion (23). Oxidative stress in a tissue like testis, with its high rates of metabolism and cell replication, can be especially damaging. In addition, testicular cell membranes are rich in polyunsaturated fatty acids and thus susceptible to oxidative injury (24). As might be expected, inhibitors of oxidative stress provide a significant beneficial effect on testicular IR injury (25). Studies have shown that antioxidants play a critical role in the treatment of IR injury (11,12). Ranade et al. showed that a potent antioxidant like vitamin E provides significant protection against acute testicular torsion and detorsion injury (3). The antioxidative effect of ghrelin has been described earlier. Kheradmand et al. indicated that administration of ghrelin to rats not suffering from induced oxidative stress also improved testicular total antioxidant capacity (26). Actually, the protective effect of ghrelin in I/R damages of other tissues such as gastric mucosa (11) and heart (27) by its antioxidant activity is well-known. In the previous study, we showed that pretreatment with ghrelin in testicular I/R group reversed the MDA level (an end product of lipid peroxidation) in the ipsilateral testis to control, and significantly enhanced antioxidant enzyme GPx activity (12). GPx is an important antioxidant enzyme that rapidly converts H2O2 to water and prevents the accumulation of H2O2 (22). As the accumulation of H2O2 leads to lipid peroxidation, we proposed that ghrelin has decreased the lipid peroxidation by enhancement of GHx activity (12). The reaction of the superoxide radicals with nitric oxide molecules to form peroxynitrite which promotes further damage to reperfused tissues is the alternative way in IR-induced injury (28).

It is worth to note that besides oxidative damage, a high apoptotic cell death may play significant roles in the cellular injury caused by various ulcerogenic (29). Emerging data indicate that both distinct types of cell death, necrosis and apoptosis may take place simultaneously in oxidative conditions like IR injury (30). Oxidative stress could be a putative mediator of apoptosis by decreasing intracellular glutathione, the major buffer of the cellular redox status and/or by increasing cellular reactive species (31,32). H2O2 at low doses induces apoptosis via production of OH radicals and alteration of the oxidant/antioxidant pathway (33). Oppositely, antioxidants can inhibit a variety of the apoptotic pathways (34). Increased activity of GPx leads to a change in redox ratio (reduced / oxidized forms) in favor of reduced glutathione. This ratio is a useful measurement for determining oxidative stress and

changes in this ratio appear to correlate with cell proliferation, differentiation, and apoptosis (35). Previously it has been indicated that excessive ROS may elicit the induction of apoptosis in testicular germ cells in IR (1). Beheshtian *et al*., prevented I/R-induced testicular germ cell apoptosis by supplementation of an antioxidant as sildenafil (36). The possible involvement of ghrelin in the protection of numerous tissues against IR-induced apoptosis has been suggested (37). Recently, our co-workers showed that ghrelin modulates testicular germ cells apoptosis and proliferation in adult normal rats (38). Taken together, it is possible to postulate that in the present study, restoration of tissue structure by pretreatment of rats with ghrelin prior to detorsion may be attributable to antioxidant properties of ghrelin.

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