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Ghrelin is a suppressor of testicular damage following experimentally induced cryptorchidism in the rat

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ABSTRACT

Background: Cryptorchidism is associated with increased level of reactive oxygen species and lipid peroxidation. This study was undertaken to examine the possible ghrelin ability in attenuation of testicular damage in response to elevated temperature.
Methods: Thirty male rats were subdivided into sham-operated, cryptorchidism-saline and cryptorchidism-ghrelin group. Bilateral cryptorchidism was induced in groups 2 and 3, surgically. The animals in group 3 were given ghrelin for 7 days and all testes were taken for biochemical and photomicrograph analysis.
Results: Glutathione peroxidase activity and glutathione content significantly promoted on day 7 in the cryptorchid rats treated by ghrelin. Catalase activity was higher in the ghrelin-exposed animals than the cryptorchidism-saline group on both experimental days. Although superoxide dismutase activity was elevated by ghrelin treatment on both days, it did not differ significantly. By contrast, significant reduction was observed in thiobarbituric acid reactive substances concentrations following ghrelin administration on day 7. Moreover, ghrelin could improve histopathological scores of the testes, and diminished formation of giant cells and tubular vacuolization.
Conclusions: These findings indicate for the first time the novel evidence of ghrelin antioxidant properties in attenuation of rat testicular injury following experimentally induced cryptorchidism.

Mammalian testes descend down into extraabdominal scrotal sac during fetal development where temperature is optimal for spermatogenesis. Failure of this process leads to cryptorchidism, as a congenital abnormality [1]. Cryptorchidism is a serious problem, which affects 2%–5% of the male population [2,3]. Failure of testes to descend into the scrotal region impairs germ cell development and subsequent arrest of spermatogenesis in response to elevated abdominal temperature. This is associated with male infertility as well as a greater incidence of testicular cancer [3–5]. Various studies indicate that testicular germ cell loss following cryptorchidism is mediated by apoptosis, so that primary spermatocytes and spermatids are the most susceptible cells during heat stress [2,5–11].

It has been indicated that reactive oxygen species (ROS) may contribute in the induction of germ cell apoptosis in cryptorchid testes [12]. In this sense, it has been well documented that impaired detoxification of ROS results in the oxidative stress and increased peroxidation of cellular lipids in abdominal position of the testes [11,13,14]. To further support, it has been shown that germ cell apoptosis in cryptorchidism is related closely to the decrease of antioxidant enzyme activities [13,15].

The role of ROS in rat testicular germ cell apoptosis following heat stress and the protective effects of various antioxidants have been fully described [16,17], which demonstrated that heat stress induces an elevation of intracellular peroxide levels as early as 5 min after exposure to 43 ºC and showed the possibility that elevation of intracellular peroxide levels may be a signal triggering apoptosis.

It has been recently proposed that ghrelin is an endogenous antioxidant and functions as a free radical scavenger [18]. The antioxidant properties of ghrelin via increasing of antioxidant enzyme activities and reduction in lipid peroxidation have been newly reported in our laboratory in the rat normal testis [19,20] and ovary [21]. More recently, we have reported that ghrelin could significantly down-regulate Bax expression in both spermatocytes and spermatogonia following scrotal hyperthermia during immersion of testis in water bath at 43 ºC [22]. Therefore it is expected that antioxidant agents may inhibit the adverse effects of ROS-induced germ cells degeneration in the cryptorchidism.

With the regard to previous studies, we designed the present investigation to explore the possible ghrelin efficacy in protection of testicular cells injury against oxidative stress following surgically induced cryptorchidism in the rat.

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1. Materials and methods

1.1. Animals

The experiment was carried out on 30 adult male Wistar rats weighing 200–220 g bred in the vivarium of Razi Herbal Medicine Research Center, Khorram Abad, Iran. 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 (Khorram Abad, Iran). The rats were housed (five rats per cage) in animal room under constant 12-h light/12-h darkness cycle and controlled temperature (21–24 °C) conditions. All animals were allowed free access to standard laboratory rat chow and tap water ad libitum.

1.2. Reagents and drugs

The kits used for the measurement of antioxidant enzyme activities were provided from Randox Laboratories Ltd. (Antrim, UK). Other chemicals were purchased from Sigma-Aldrich Company (St. Louis, MO) unless otherwise indicated. Rat-lyophilized acylated ghrelin (*n*-octanoylated research grade) was purchased from Tocris Cookson Ltd. (Bristol, UK). Ghrelin was dissolved in sterile physiologic saline solution before injection.

1.3. Experimental design and surgical protocol

The animals were randomly divided into three equal groups (each containing 10 rats) as the following: group 1 was served as sham-operated or control-saline (CS), group 2 was defined as cryptorchidism-saline (CrS) and group 3 was assumed as cryptorchidism-ghrelin (CrG) animals. Surgical procedure for induction of bilateral cryptorchidism was performed according to the method of inguinoscrotal approach as previously described by Dundar et al. [23]. General anesthesia was achieved intraperitoneally by a combination of ketamin (Alfasan, Woerden, Holland, 70 mg kg^{-1}) and xylazine hydrochloride (Alfasan, Woerden, Holland, 5 mg kg^{-1}). After anesthesia, the scrotal area was shaved and prepared by povidone iodine solution. In groups 2 and 3, inguinoscrotal region was incised and gubernaculum was separated where it protruded from the abdominal wall and then the external inguinal ring was revealed. After pushing the gubernaculum into the abdominal cavity, the external inguinal ring and inguinoscrotal wall were closed by 2/0 simple silk suture. During the sham operation, the both testes were brought through the incision and then replaced after a few manipulations. Immediately after surgery, CrG rats were given ghrelin subcutaneously (10 nmol/100 μl saline) for 7 consecutive days. The animals in CS and CrS groups were treated by sterile saline instead of ghrelin as the same method. The dose of ghrelin used in our in vivo setting was close to the dose applied in our recent experiment as a pretreatment for protection of testicular tissue against oxidative damages induced by ischemia/reperfusion in the rat [24]. The animals were injected under conscious conditions after careful handling to avoid any stressful influence. Five rats from each group were killed upon diethyl ether anesthesia (Merck, Darmstadt, Germany) by decapitation on days 3and 7 after surgery.

1.4. Sampling and tissue preparation

Immediately after rat killing on days 3 and 7, the left testis in each group was removed and carefully cleaned of fat and adhering then stored at liquid nitrogen prior to analysis for testicular antioxidant enzymes activity and lipid peroxidation. Just before measurements, the testis was rapidly thawed and manually homogenized in cold phosphate buffer (pH7.4) and debris were removed by centrifugation at 3500 *g* for 10 min (Rotofix 32 A, Hettich, Tuttlingen, Germany). The upper clear supernatants were recovered for enzyme and protein assays. The right testis was also taken and fixed in Bouin's solution for later histopathological assessment.

1.5. Biochemical estimations

1.5.1. Lipid peroxidation assay

The amount of lipid peroxidation was determined spectrophotometrically by following the production of thiobarbituric acid reactive substances (TBARS) as described by Subbarao et al. [25] which has been more explained in our previous work [21]. TBARS results were expressed as nanomoles per milligram of tissue protein (nmol/mg protein).

1.5.2. Antioxidant enzymes assay

Total superoxide dismutase (SOD) activity was evaluated by SOD detection kit according to the manufacturer's instructions. The role of SOD is to accelerate the dismutation of the toxic superoxide (O_2^-) produced during oxidative energy processes to hydrogen peroxide and molecular oxygen. SOD activity was recorded at 505 nm and through a standard curve and expressed as unit per milligram of protein (U/mg protein).

The activity of glutathione peroxidase (GPx) was evaluated by GPx detection kit according to the manufacturer's instructions. GPx catalyzes the oxidation of glutathione (GSH) by cumene hydroperoxide. The decrease in absorbance at 340 nm against blank was measured using multicell changer spectrophotometer (Jenway, 6715 UV/Vis., Bibby Scientific Ltd., Dunmow, Essex, UK). The GPx activity was expressed as miliunit per milligram of tissue protein (mU/mg protein).

Tissue catalase (CAT) activity was assayed using the method described by Claiborne [26] and our earlier technical explanations [21]. The CAT activity was expressed as the unit that is defined as μmol of H_2O_2 consumed per minute per milligram of tissue protein (U/mg protein).

1.5.3. Total GSH content

Total glutathione (GSH) was estimated by the model of Sedlak and Lindsay [27] which was fully described in our previous report [21]. Both the blank and sample reaction mixtures were read against water at 412 nm. GSH concentration was calculated on the basis of a millimolar extinction coefficient of 13.6 and a molecular weight of 307 g.

1.5.4. Protein assay

Protein content of tissue homogenates was determined by a colorimetric method of Lowry using bovine serum albumin as standard [28].

1.6. Histopathological evaluations

The samples were fixed and 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 μm on a rotary microtome, mounted on slides and stained with hematoxylin–eosin (H&E) and examined under a light microscope. Evaluation of testicular damage and impaired spermatogenesis was conducted by an expert pathologist who was blind to the sample groups and also was graded as described by Johnsen [29]. Briefly, a score of 1 indicated no seminiferous epithelial cells and tubular sclerosis. A score of 2 indicated no germ cells, only Sertoli cells. A score of 3 indicated spermatogonia only. A score of 4 indicated no spermatids, few spermatocytes, and arrest of spermatogenesis at the primary spermatocyte stage. A score of 5 indicated no spermatids and many spermatocytes. A score of 6 indicated no late spermatids,