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Biochemical and histopathological evaluations of ghrelin effects following cadmium toxicity in the rat testis

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Summary

Numerous reports demonstrate that cadmium (Cd) induces oxidative stress by increasing lipid peroxidation and altering antioxidative enzymes status. Thirty male rats were subdivided into control-saline, Cd-saline and Cd-ghrelin groups. A single dose of Cd was injected to induce testicular injury and also ghrelin for 10 consecutive days to group 3. SOD activity decreased and lipid peroxidation increased by Cd administration. The mean activities of GPx and CAT as well as GSH content were lower in the Cd-saline rats; however, they did not statistically differ compared with the controls. Exposure to Cd resulted in complete degeneration of seminiferous tubules with severe depletion of germ cells and arrest in spermatogenesis. Notably, ghrelin treatment not only prevented reduction in SOD, GPx, CAT and GSH level, but also increased enzyme activities form their normal values. Moreover, TBARS concentration was significantly reduced by ghrelin administration. Furthermore, ghrelin pre-treatment resulted in partial but not significant prevention in testicular histopathological features damaged by Cd. In conclusion, the obtained results indicate for the first time the novel evidences of ghrelin ability in promotion of antioxidant enzyme activities and reduction of lipid peroxidation following Cd-induced oxidative stress in the rat testis. These observations also demonstrate that ghrelin may be considered as promising antioxidant agent in prevention and attenuation of testicular injury upon Cd toxicity.

Introduction

Cadmium is a well-recognised environmental pollutant with several adverse health effects (Xu *et al.*, 2003). This heavy and potent toxic metal is very harmful to humans and animals because of its *in vivo* accumulation in tissues, thus causing pathological and biochemical changes (Waisberg *et al.*, 2003; Thompson & Bannigan, 2008). Various organs including kidney, liver, embryo and reproductive tissues are affected by Cd; however, it is well established that the testis is exceedingly sensitive to Cd toxicity in both animal and human populations (Oldereid *et al.*, 1993; Goyer *et al.*, 2004; Siu *et al.*, 2009).

It has been generally accepted that the pathogenesis of testicular damage upon Cd exposure is generation of reactive oxygen species (ROS). Decreased activity of antioxidant enzymes including SOD, GPx and CAT and subsequent increased lipid peroxidation strongly suggests that Cd induces oxidative stress in the testis (Agarwal *et al.*, 1997; Siu *et al.*, 2009). Therefore, it is potentially expected that testis could be protected against toxic effects of Cd by antioxidant treatment. In this regard, many investigations have focused on the treatment of testis by some antioxidant agents such as vitamin C, E, heparin or Q_{10} during Cd poisoning (Agarwal *et al.*, 1997; Sen Gupta *et al.*, 2004; Koyuturk *et al.*, 2006; Yang *et al.*, 2006; Kara *et al.*, 2007; Amara *et al.*, 2008; Messaoudi *et al.*, 2010; Ognjanovic *et al.*, 2010).

Ghrelin, the endogenous ligand for the growth hormone secretagogue receptor, has been shown to increase antioxidant defence system and inhibit lipid peroxidation in different organs including brain (Obay *et al.*, 2008), adipocyte tissue (Zwirska-Korczala *et al.* 2007), stomach (Iseri *et al.*, 2005) and also hypertensive rats (Kawczynska-Drozdz *et al.*, 2006). Antioxidant properties of ghrelin have been demonstrated in our laboratory in the rat

normal testis (Kheradmand *et al.*, 2009a) and ovary (Kheradmand *et al.*, 2010).

In the light of these literatures, it is reasonable to assume that ghrelin may protect testicular tissue against Cd-induced oxidative stress. Thus, biochemical and pathological characteristics were analysed to clarify the possible beneficial role of ghrelin following Cd-induced testicular lesion.

Materials and methods

Animals

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 experiment was performed on thirty adult male Wistar rats weighing 200–220 g bred in the vivarium of Razi Herbal Medicine Research Center, 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 and had free access to a pelleted food and tap water *ad libitum.*

Drugs and reagens

Rat lyophilised acylated ghrelin (n-octanoylated research grade) were purchased from Tocris Cookson Ltd. (Bristol, UK). Ghrelin was dissolved in sterile physiologic saline solution before injection. Cadmium chloride was supplied from Sigma-Aldrich Company (St. Louis, MO, USA). The kits that were used for the measurement of antioxidant enzyme activities were provided from Randox Laboratories Ltd. (Antrim, UK).

Experimental design

The animals were randomly allocated into the following three groups ($n = 10$ in each group): control-saline, cadmium-saline and cadmium-ghrelin. Induction of testicular injury was achieved by a single injection of cadmium chloride solution at the dose of 2 mg kg^{-1} intraperitoneally to groups 2 and 3. The dose of Cd was chosen according to the investigation of Sen Gupta *et al.* (2004). Higher doses of Cd has been shown to induce irreversible and massive destruction of testicular germ cells 1 day after the injection (Ikemoto *et al.*, 1990), and this extensive testicular damage cannot be prevented by free radical scavengers at their common doses (Agarwal *et al.*, 1997; Yang *et al.*, 2006). Group 3 was given ghrelin subcutaneously (10 nmol/100 μl saline) 1 day before being challenged with cadmium and continued for 10 consecutive days. The dose of ghrelin used in our *in vivo* experiment was close to the dose applied in our recent work as a pre-treatment for protection of testicular tissue against acute oxidative damages induced by ischaemia/reperfusion in the rat (Taati *et al.*, 2012). The animals in the control and Cd-saline groups were treated by sterile saline instead of ghrelin as the same method. The animals were injected under conscious conditions after careful handling to avoid any stressful influence. Five rats from each group were sacrificed upon diethyl ether anaesthesia (Merck, Darmstadt, Germany) on days 5 and 10 after Cd injection.

Sampling and tissue preparation

Immediately after rat killing on days 5 and 10, 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 enzyme activities and TBASR content. Just before measurements, the rat testis was rapidly thawed and manually homogenised in cold phosphate buffer (pH 7.4) and debris removed by centrifugation (Rotofix 32 A, Hettich, Tuttlingen, Germany) at 3500 ***g*** for 10 min. 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 analysis.

Biochemical measurements

Lipid peroxidation assay

The amount of lipid peroxidation was determined by following the production of TBARS as described by Subbarao *et al.* (1990). In short, 40 μl of homogenate was added to 40 μl of 0.9% NaCl and 40 μl of deionised H_2O, resulting in a total reaction volume of 120 μl. The reaction was incubated at 37 °C for 20 min and stopped by the addition of 600 μl of cold 0.8 M hydrochloride acid (HCL), containing 12.5% trichloroacetic acid (TCA). Following the addition of 780 μl of 1% TBA, the reaction was boiled for 20 min and then cooled at 4 °C for 1 h. To measure the amount of TBARS produced by the homogenate, the cooled reaction was spun at 1500 ***g*** in a microcentrifuge for 20 min and the absorbance of the supernatant was spectrophotometrically read at 532 nm, using an extinction coefficient of 1.56×10^5 M cm^{-1}. The blanks for all of the TBARS assays contained an additional 40 μl of 0.9% NaCl instead of homogenate as just described. TBARS results were expressed as nanomoles per milligram of tissue protein (nmol mg^{-1} protein).

Antioxidant enzymes assay

Total 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. This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. The SOD activity is then measured by degree of inhibition of this reaction. One unit of SOD is that which causes 50% inhibition of the rate of reduction in INT under the conditions of the assay. SOD activity was recorded at 505 nm and through a standard curve and expressed as unit per milligram of protein (U mg^{-1} protein).

The activity of GPx was evaluated by GPx detection kit according to the manufacturer's instructions. GPx catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, the oxidised glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm against blank was measured using multi-cell changer spectrophotometer (Jenway, 6715 UV/Vis., Bibby Scientific Ltd., Dunmow, Essex, UK). One unit (U) of GPx activity was defined as amount of enzyme that converts one μmol of NADPH to $NADP^+$ per minute. The GPx activity was expressed as milliunit per milligram of tissue protein (mU mg^{-1} protein).

Tissue CAT activity was assayed using the method described by Claiborne (1986). The reaction mixture (1 ml) consisted of 50 mM potassium phosphate (pH 7.0), 19 mM H_2O_2 and a 20–50 μl sample. The reaction was initiated by the addition of H_2O_2, and absorbance changes were measured at 240 nm (25 °C) for 30 s. The molar extinction coefficient for H_2O_2 is 43.6 M cm^{-1}. The CAT activity was expressed as the unit that is defined as μmol of H_2O_2 consumed per min per milligram of tissue protein (U mg^{-1} protein).

GSH level

Total GSH content was estimated by the model of Sedlak & Lindsay (1968). Briefly, 5% tissue homogenates were prepared in 20 mM EDTA, pH 4.7, and 100 μl of the homogenate or pure GSH was added to 0.2 M Tris-EDTA (1.0 ml, pH 8.2) buffer (Fluka, St. Gallen, Switzerland) and 20 mM EDTA, pH 4.7 (0.9 ml) followed by 20 μl of Ellman's reagent (10 mM DTNB in methanol). After 30 min of incubation at room temperature, absorbance was read at 412 nm. The blank was prepared with 1 ml phosphate solution, 1 ml water, 0.5 ml precipitating solution and 250 μl DTNB solution. 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.

Protein measurement

Protein content of tissue homogenates were determined by a colorimetric method of Lowry using bovine serum albumin as standard (Lowry *et al.*, 1951).

Histopathologiacl assessment

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 haematoxylin–eosin (H&E) and examined under a light microscope. Evaluation of testicular damage and impaired spermatogenesis was graded as described by Johnsen (1970). 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, few early spermatids, arrest of spermatogenesis at the spermatid stage and disturbance of spermatid differentiation. A score of 7 indicated no late spermatids and many early spermatids. A score of 8 indicated few late spermatids. A score of 9 indicated many late spermatids and disorganised tubular epithelium. A score of 10 indicated full spermatogenesis.

Statistical analysis

Results were analysed using SPSS/PC program (SPSS Inc., Chicago, IL, USA). All data were tested for normality followed by *Levene's static test* for homogeneity of variances. When the variances were homogenous, the activities of SOD, GPx, CAT, GSH content and TBARS levels as well as histopathological grades on days 5 and 10 among three groups were compared using *one-way* ANOVA and *Tukey's test* as *post hoc*, to determine the difference among groups (Petrie & Watson, 1999). Data are presented as the mean ± SEM, and significant level was set at $P < 0.05$.

Results

Biochemical parameters

Exposure of rats to Cd resulted in significant reduction in SOD activity compared with the control group on day 5 (Fig. 1, $P < 0.05$). But, despite suppression in GPx and CAT activities in the Cd-saline rats, however, their values did not still exhibit differences compared with the control animals. Moreover, Cd toxicity prominently diminished testicular GSH content on days 5 and 10, but again, the differences was not still statistically significant when compared with the control (Fig. 1, $P > 0.05$). In contrast, acute single injection of Cd caused remarkable increase in lipid peroxidation levels on both experimental days ($P < 0.001$).

Interestingly, cotreatment of Cd with ghrelin restored testicular antioxidant status, by which significant increments were seen in all of enzyme activity values by day 10 in comparison with the Cd-saline group (Fig. 1). On the other hand, lipid peroxidation marker, TBARS level, was reduced by ghrelin therapy either on day 5 or on day 10 ($P < 0.05$, Fig. 2). As shown in Figure 1, cellular GSH content was also proceeded from its normal value by ghrelin administration, so that it caused to be higher than the Cd-exposed group ($P < 0.01$).

Histopathological assessment

Light microscope examination revealed extensive epithelial destruction of seminiferous tubules upon Cd injection. Seminiferous epithelium was devoid of most germinal layer, associated with impaired spermatogenesis and inter-tubular haemorrahage (Fig. 3). Among germ cells, only spermatogonia were detected in some of tubules and other cell forms have been disappeared. As indicated by Johnsen's grade (Tables 1 and 2), the mean value of tubular compartment was approximately 2.5 in Cd-saline group, which indicates that just a few tubules rimmed by spermatogonia. It should be considered that although ghrelin treatment ameliorated Cd-induced testicular injury and caused partial reversal in tubular architecture associated with some evidences of active spermatocytes, however, neither on day 5 nor on day 10,

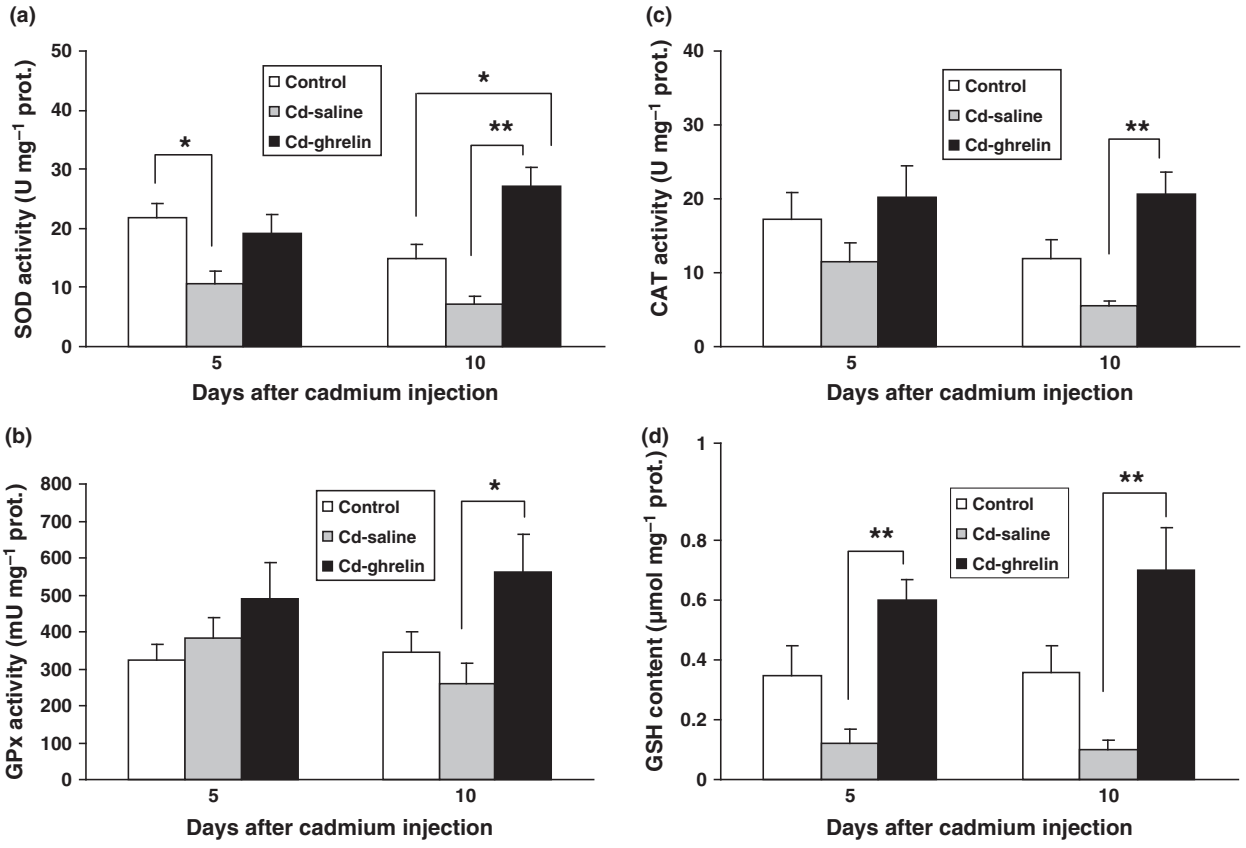

Fig. 1 Testicular SOD (a), GPx (b) and CAT (c) activities as well as GSH content (d) on days 5 and 10 after Cd toxicity. Values represent mean ± SEM in each group. All means marked with *($P < 0.05$) and **($P < 0.01$) are significantly different from each other.

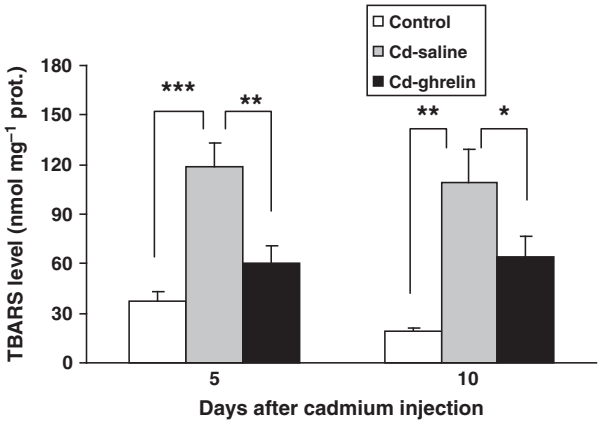

Fig. 2 Testicular TBARS levels on days 5 and 10 after Cd administration. Values represent mean ± SEM in each group. All means marked with *($P < 0.05$), **($P < 0.01$) and ***($P < 0.001$) are significantly different from each other.

their scores showed statistical differences compared with the Cd-saline group. In other words, histopathologiacl features of testis in the Cd-exposed rats did not improve promptly by concurrent ghrelin treatment for 10 days. Such a similar observation was also observed in the percentage of seminiferous tubules containing multinuclear giant cell, by which ghrelin administration could not significantly reduce the percentage of tubules represent theses cells.

Discussion

This study probably is the first demonstrating that treatment by ghrelin could strongly promote antioxidant enzymes capacity following testicular injury induced by Cd. Moreover, ghrelin administration normalised the increased TBARS concentration caused by Cd exposure. These actions of ghrelin are mainly mediated through its antioxidant properties, which can prevent Cd-induced oxidative damage. Moreover, ghrelin therapy resulted in partial but not significant prevention in histopathological features of testis after serious degeneration by Cd.

Cadmium is a toxic metal, which enhances oxidative stress and contributes to the development of severe

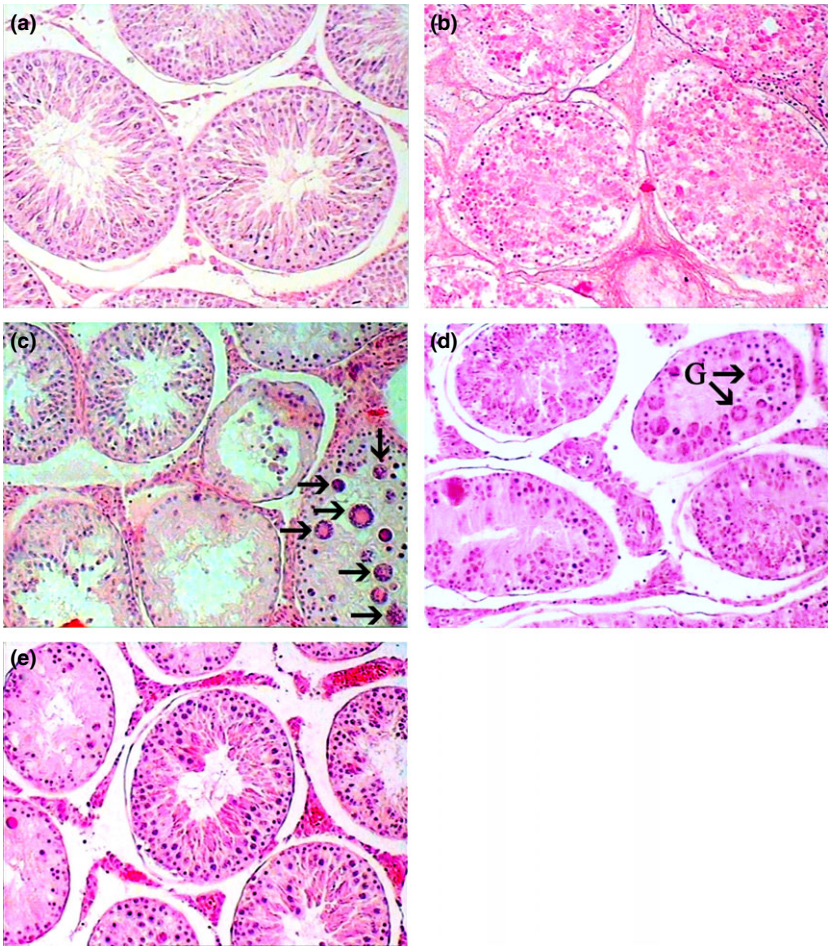

Fig. 3 Testicular architecture in the control normal rats (a) with well-organised distribution cells and full spermatogenesis. (b, c): Extensive degeneration of germinal epithelium associated with arrest of spermatogenesis in Cd-exposed animals on days 5 (b) and 10 (c). Multinuclear giant cells are depicted by arrows. (d, e): Partial regeneration of histopathological features of testis following ghrelin administration. Multinuclear giant cells are still present on day 5 (d); however, some evidences of tubular regeneration are seen at days 10 (e). G: giant cell (400×).

Table 1 Mean ± SEM of histopathological parameters on day 5 after Cd toxicity in all experimental groups

Groups	Johnsen score	Giant cells (%)
Control-saline	9.60 ± 0.24	0.00
Cd-saline Cadmium-saline	2.40 ± 0.25**	28.0 ± 7.36*
Cd-ghrelin Cadmium-ghrelin	2.84 ± 0.41**	37.71 ± 6.28**

Statistically different from the control group, *$P < 0.05$, **$P < 0.001$.
Statistically different from the Cadmium-saline.

Table 2 Mean ± SEM of histopathological parameters on day 10 after Cd toxicity in all of experimental groups

Groups	Johnsen score	Giant cells (%)
Control-saline	9.80 ± 0.20	0.00
Cd-saline Cadmium-saline	2.61 ± 0.54**	23.33 ± 7.61*
Cd-ghrelin Cadmium-ghrelin	3.78 ± 0.75**	29.5 ± 1.18*

Statistically different from the control group, *$P < 0.05$, **$P < 0.001$.
Statistically different from the Cadmium-saline.

degenerative changes in several tissues (Koyuturk *et al.*, 2006). It is postulated that the mammalian testis is more sensitive to Cd than the other organs, because of its unique vasculature (Aoki & Hoffer, 1978; Siu *et al.*, 2009). It is believed that increased generation of ROS and subsequent oxidative stress induced by Cd has a causative role in the pathogenesis of testicular injury (Sen Gupta *et al.*, 2004; Kara *et al.*, 2007; Siu *et al.*, 2009). Phagocytic cells may be an important source of ROS in response to Cd ions (Stohs & Bagachi, 1995). In addition, Cd could displace Fe from its binding sites, leading to Fe redistribution and the generation of ROS via Fenton chemistry, which may then give rise to lipid peroxidation (Casalino *et al.*, 1997).

There are numerous reports concerning Cd influence on the various antioxidant enzyme activities and the beneficial effects of different antioxidant agents in amelioration of Cd toxicity. However to date, there is no available data about ghrelin protective ability upon Cd-induced testicular damage. For instance, it have been shown that Cd decreases testicular SOD or GPx (Sen Gupta *et al.*, 2004; Kara *et al.*, 2007; Messaoudi *et al.*, 2010) and CAT activities (Amara *et al.*, 2008; Ognjanovic *et al.*, 2010) as well as glutathione level (Koyuturk *et al.*, 2006). All of the above investigations also confirmed the increased lipid peroxidation level after Cd intoxication. Similar results were also obtained in the present work: reduction of enzyme activities and increment of TBARS concentration after Cd injection. Clearly, reversal in all of enzymatic activities and minimising of lipid peroxidation were observed after co-administration of ghrelin. These observations suggest that ghrelin might preserve testicular tissue against Cd toxicity by improvement of antioxidant enzymes status and subsequent reduction of lipid peroxidation, as a known marker of oxidative stress.

The interaction between Cd and essential trace elements could be one of the reasons for decreased enzymatic activity in the rat testis. Cd can displace iron (Fe) from its binding sites, as CAT contains Fe in its active centre; therefore, decline in the activity of CAT might be a result of Fe deficiency. On the other hand, Cd is able to occupy the site of zinc (Zn) in zinc/cooper SOD molecule, which creates an inactive form of this enzyme (Bauer *et al.*, 1980). Plasma Zn depletion associated with Cd injection also supports the causative role of Cd in SOD decreased activity. GSH is the most abundant cellular thiol, which serves to protect against various forms of metal toxicity (Dalton *et al.*, 2004). GSH is also known as free radical scavenger and potent inhibitor of lipid peroxidation (Arthur, 2000). It has been proved that Cd induces GSH depletion with concomitant ROS generation (Bagchi *et al.*, 1996). It was also indicated that GSH concentration might be direct or indirect targets for Cd toxicity in the testis (Bagchi *et al.*, 1996). Accordingly, lower level of GSH on days 5 and 10 in Cd-saline group in the current study is in conjunction with previous reports and therefore could be justified. Notably, ghrelin-treated rats demonstrated higher levels of GSH, so that it was more pronounced than those observed in two other groups.

Our results clearly showed that the mean activities of SOD, GPx and CAT enhanced by ghrelin cotreatment. SOD rapidly converts superoxide anion (O_2^-) to less dangerous hydrogen peroxide (H_2O_2). GPx and CAT can decompose H_2O_2 to water. H_2O_2 is not a particularly reactive product, but it may be reduced to the highly reactive metabolites hydroxyl radicals (OH^-) or single oxygen (Peltola *et al.*, 1992). Decreased SOD activity may imply reduced H_2O_2 production followed by subsequent decline in the CAT activity (Kono & Fridovic, 1982). It is well documented that the activity of CAT is directly proportional to the substrate level assumed to be produced by SOD (Patra *et al.*, 1999; Aitken & Roman, 2008).

On the other hand, treatment of animals exposed to Cd with ghrelin significantly increased activity of GPx and GSH content. Of particular note, GPx is highly dependent on glutathione concentration. Cd binds to cysteine in reduced glutathione (GSH), resulting in the inactivation of GPx which therefore fails to metabolise H_2O_2 to water. Moreover, there is increasing evidence that Cd interacts with Se and disrupts GPx activity (Acharya *et al.*, 2008). GSH is an important antioxidant defence, which forms complexes with Cd through the free –SH group and thereby changes Cd distribution (Singhal *et al.*, 1987). GSH also acts as a direct free radical scavenger and is a cosubstrate for GPx activity (Griffth, 1999). It

has been clearly showed that lipid peroxidation (evaluated by TBARS value) significantly increases by accumulation of H_2O_2 as a concentration-dependent manner (Garcia *et al.*, 2005). The principal mechanism of H_2O_2 toxicity is thought to be involved in the generation of highly reactive radical ($OH^{\cdot}$) through interaction with Fe^{2+} by the Fenton reaction (Sewerynek *et al.*, 1995). It seems that the increase in GPx activity in the present study causes more rapid conversion of H_2O_2 to H_2O and preventing of H_2O_2 accumulation to shift for lipid peroxides production. This is further supported by the fact that we detected lower TBARS content in the rat testis following treatment by ghrelin. Furthermore, it is believed that GPx functions in the detoxification of reactive lipid peroxides (Peltola *et al.*, 1992) and therefore, the reduction in TBARS concentration after ghrelin application can be justified in the present work.

The presence of different antioxidant enzymes have been investigated in the rat testis. As compared with the liver, the rat testis expressed equivalent levels of SOD activity but only 5% of the GPx activity and 2% of CAT activity (Peltola *et al.*, 1992), and as compared to somatic cells, such as Sertoli or peritubular cells, the testicular germ cells presented low glutathione-dependent enzyme activity (Bauche *et al.*, 1993). These data indicate a low capacity for scavenging hydrogen peroxide in the testicular germ cells. Recently, antioxidant properties of ghrelin have been demonstrated in our laboratory in the rat testis (Kheradmand *et al.*, 2009a) and ovary (Kheradmand *et al.*, 2010) so that ghrelin was able to promote antioxidant enzyme activities (especially GPx) and reduce lipid peroxidation in the testis. In addition, we indicated that chronic administration of ghrelin increases functional membrane integrity of rat spermatozoa (Kheradmand *et al.*, 2009b). Likewise, the antioxidant properties of ghrelin are in consistent with our another study, in which we have shown that ghrelin enhances viability of rat spermatozoa during incubation at 37 °C up to 5 h, because of its antioxidant characteristics (Kheradmand *et al.*, 2009c).

The Cd-induced hypocellularity observed in the present study is agreement with the other investigations (Yang *et al.*, 2006; Kara *et al.*, 2007). The most of tubules exposed to Cd exhibit massive germ cells loss, and only spermatogonia remained in some of tubules to cause spermatogenesis resumption after Cd removal. It is thought that ROS are responsible for testicular degeneration in Cd toxicity (Mathur *et al.*, 2011). Oxidative stress can conflict direct oxidative damage to genomic DNA or upregulate apoptotic proteins, which leads to germ cell loss and impaired spermatogenesis (Saradha & Mathur, 2006). Oxidative stress and the release of ROS have been linked by many studies for description of germ cell apoptosis and are a direct inducer of apoptosis in the testis (Ikeda *et al.*, 1999; Erkkila *et al.*, 1999; Doreswamy *et al.*, 2004).

Although testicular injury may be prevented by ghrelin administration via promotion of antioxidant enzyme activities and suppression of lipid peroxidation, however, 10 days treatment could not significantly reverse histological features of testis. The beneficial effects of ghrelin pre-treatment in improvement of antioxidant enzyme activities after 4 h of reperfusion was observed previously (Taati *et al.*, 2012), Therefore, due to the high toxicity of Cd for testis, we set the present experiment for up to 10 days as pre-treatment protocol. However, 10 days of ghrelin treatment with the same dose was not able to change testicular architecture, possibly due to the more oxidative stress induced by Cd. Likely, a prolong time is needed for possible repair in testicular cytoarchitecture. This hypothesis is completely in consistent with our previous report, by which ghrelin significantly stimulated testicular regeneration just after 30 days upon oxidative stress induced by local scrotal hyperthermia (Kheradmand *et al.*, 2011). It seems that despite pre-treatment by ghrlin with higher dose in the current study compared with our earlier study, 10 days is too short period and is not sufficient for protecting of degenerated seminiferous tubules after severe toxicity of Cd, and therefore, further time is required for testicular regeneration.

It has been demonstrated that intratesticular injection of ghrelin in adult rats inhibited expression of the gene-encoding stem cell factor (SCF), a key signal in spermatogenesis and putative regulator of Leydig cell development. (Barreiro *et al.*, 2004). Notably, SCF is a Sertoli cell product that has been identified as the major paracrine stimulator of germ cell development (Garcia *et al.*, 2007). On the other hand, ghrelin was shown to suppress luteinizing hormone (LH) secretion *in vivo* and to decrease LH responsiveness to GnRH *in vitro*. Moreover, ghrelin was able to inhibit stimulated testicular testosterone secretion *in vitro* (Tena-Sempere *et al.*, 2002; Barreiro & Tena-Sempere, 2004; Fernandez-Fernandez *et al.*, 2005, 2006). Likewise, it has been proved that infusion of ghrelin throughout puberty for 10 days decreased LH levels and to adult male rats resulted in significant decreases in circulating LH and FSH concentrations (Martini *et al.*, 2006). As secretion of FSH and testosterone is necessary for the spermatogenes cycle, the obtained histopathological results in the present study might be expectable. Receptors of FSH are solely expressed in Sertoli cells, and FSH is the major regulator of testicular SCF expression (Hakovitra *et al.*, 1999). This suggests a direct effect of ghrelin on Sertoli cells, where the functional ghrelin receptor, GHS-R1a, has been detected (Barreiro *et al.*, 2004; Budak *et al.*, 2006). In fact, the action of ghrelin

upon testicular SCF mRNA expression may have implications not only in Leydig cell proliferation, but also in the control of spermatogenesis because SCF has been pointed out as the major paracrine stimulator of germ cell development, acting as a survival factor for spermatogonia, spermatocytes and spermatids in the adult rat seminiferous epithelium (Hakovitra *et al.*, 1999; Budak *et al.*, 2006).

It should be noted that although we did not explore the role of anti-apoptotic and proliferative protein expressions after cotreatment of Cd with ghrelin in the current study, however, we recently indicated that downregulation of Bax in parallel to PCNA upstream was observed by ghrelin administration following heat-induced oxidative stress (Kheradmand *et al.*, 2012). Further investigations are necessary to elucidate the possible action of apoptotic and anti-apoptotic substances in Cd-exposed animals treated by ghrelin.

In conclusion, the present results demonstrated the novel function of ghrelin in promotion of antioxidative defence system following testicular damage in Cd-exposed rats, which appears to be mediated through its antioxidant properties. However, it could not improve testicular degeneration during the experimental period probably due to its male reproductive negative effects (Kheradmand *et al.*, 2009d). This could be potentially implicated that ghrelin might be useful in protection of testis against Cd toxicity.

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