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Tramadol reduces testicular damage of ischemia-reperfusion rats

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Abstract

The main purpose of this study was to determine effect of tramadol administration on testis histology and oxidative stress experimental on testicular ischemia-reperfusion injury in male Wistar rats. Twenty-four male Wistar rats were randomly divided into four experimental groups. The Sham group (A): no medication was employed; abdominal cavity was opened but no ischemia-reperfusion-induced. The ischemia-reperfusion group (B): abdominal cavity was opened, testicular ischemia-reperfusion-induced without pre-medication. Ischemia-reperfusion +20 mg/kg tramadol group (C), animal orally administrated with Tramadol (20 mg/kg) for 1 week prior testicular ischemia-reperfusion. Ischemia-reperfusion +40 mg/kg tramadol group (D) was similar to group C, but the animals received 40 mg/kg tramadol instead of 20 mg/kg. In all experimental groups, animals were exposed to midline laparotomy with occlusion of the infrarenal aortic for 1 h ischemia by 24 h of reperfusion in the left testis. After 24 h, the abdomen was opened, the left testis extracted for histopathological studies. Semen samples from caudal epididymis were collected to determine malondialdehyde, superoxide dismutase, glutathione peroxidase and total antioxidant status. According to the data, testicular ischemia-reperfusion degenerated seminiferous tubules and spermatogenesis in animals ($P < 0.05$). Administration of 40 mg/kg of tramadol protect testicular against ischemia-reperfusion injury ($P < 0.05$). Administration of 40mg/kg tramadol increased superoxide dismutase and glutathione peroxidase while diminished malondialdehyde levels in testicular ischemia-reperfusion injury ($P < 0.05$). These results suggest tramadol might be a potent agent in preventing testicular IR injury.

Keywords: ischemia-reperfusion, oxidative stress, testicular injury, Wistar.

Introduction

One of the most important disorders in the male reproduction system is testicular torsion (Wei *et al*., 2011), a common urologic emergency that occur with cuts off the blood supply to the testis. Ischemia-reperfusion (IR) injury is one of the main pathophysiologic conditions, which happens during testicular torsion of the testis (Wei *et al*., 2011). If testicular torsion is not treated within 4 to 6 h, infarction will occur and surgical detorsion is currently the only treatment and allows blood reperfusion. However, even in men who have undergone surgical detorsion within 4 to 6 h, the ipsilateral testes often becomes permanently dysfunctional (Wei *et al*., 2011). It seems, the main pathophysiology of testicular torsion-detorsion is ischemia-reperfusion injury of the testis (Wei *et al*., 2011).

This is a complex phenomenon which IR injury is characterized by an increase in reactive oxygen species (ROS; Nagakannan *et al*., 2012; Ashrafzadeh Takhtfooladi *et al*., 2015a). The ROS stimulate the release and the formation of various inflammatory mediators with powerful chemotactic potential (Ashrafzadeh Takhtfooladi *et al*., 2015a). Tramadol is a synthetic agonist of the opioidergic system which is used for the treatment of moderate to severe pain. The mechanism of tramadol analgesic action is complex, where it acts through central opioid receptors (Ahmed and Kurkar, 2014).

Many chemicals such as N-acetylcysteine, xanthine oxidase, curcumin, vitamin C and so one have been tested to attenuate IR injury in target and remote organs. Recently reported, administration of tramadol has shown to protect against IR injuries in local and remote organs (Ashrafzadeh Takhtfooladi *et al*., 2015b). However, the role of tramadol in reducing injury after IR has not been addressed yet (Ashrafzadeh Takhtfooladi *et al*., 2015b). The physiological levels of ROS antioxidants include superoxide dismutase (SOD), malondialdehyde (MDA), glutathione peroxidase (GPx) and total antioxidant status (TAS) are essential for proper function of male reproductive organ. On the other hand because of polyunsaturated fatty acids (PUAFAs) content of the spermatozoa, it is vulnerable to the attack of ROS (Chi *et al*., 2008).

Recently it has been revealed that tramadol decreased lipid peroxidation and regulates noradrenalin uptake; therefore, these therapeutic properties are used for the management of ischemia (Ashrafzadeh Takhtfooladi *et al*., 2015b). It is reported ROS levels decreased after administration of tramadol in myocardial IR in isolated rat hearts. However, the effects of tramadol on remote testicular injury caused by skeletal muscle ischemia/reperfusion are not clear (Bilir *et al*., 2007). Based on literature review, limited researches on the role of tramadol on experimental unilateral testicular IR injury in rat are found. In this regard, the aim of the current study was to determine the possible role of tramadol on testis histopathology as well as semen MDA, SOD, GPx and TAS levels in experimental testicular IR injury in rat.

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Materials and Methods

Animals

Twenty four male Wistar rats (230-250 g) were purchased from the Pasteur Institute and randomly allocated into four treatment groups. The rats were housed individually in cages and resided under standard laboratory conditions, according to European community suggestions for laboratory animals at a temperature of 21 ± 2°C, relative humidity of 55-60% and a 12 h of light period (starting at 8:00 AM; Council of European Communities, 1986). All animals had *ad libitum* access to chow pellets and fresh water. Animals were acclimatized to laboratory conditions for one week prior to experiments and each animal was used only once. All experimental procedures were carried in accordance with the Guide for the Care and Use of Laboratory Animals to Investigate Experimental Pain in Animals (Zimmermann, 1983). Animal handling and experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals by the US National Institutes of Health and the current laws of the Iranian government. All protocols for animal experiments were approved by the institutional animal Ethical Committee, Islamic Azad University, Science and Research Branch, Tehran, Iran (SBR1056-F1A, 2010).

Drugs and detection kits

Tramadol was obtained from the Alborz daru Co. Assay kits for MDA, SOD and GPx were purchased from the Randox (Randox Laboratories Ltd., Crumlin, Antrim, United Kingdom). The dose of tramadol was obtained from previous studies (El-Gaafarawi, 2006; Ahmed and Kurkar, 2014) and our pilot studies.

Experimental testicular ischemia-reperfusion injury

All surgical procedures were performed under anesthesia by intraperitoneal (i.p.) injection of 60 mg/kg ketamine hydrochloride and 10 mg/kg xylazine hydrochloride, then experimental testicular IR was created (Turner, 2001; Sahin *et al*., 2005). The upper left abdominal quadrant was approached through a midline laparotomy incision. During the surgical procedures, the body temperature was maintained with a heating pad. The testicular artery and vein of the left testis were occluded with a vascular clamp for 1 h, after this process the clamp was removed and the organ was allowed to reperfusion 24 h (Koksal *et al*., 2012). At the end of the study, rats were euthanized with an overdose injection of pentobarbital (300 mg/kg, i.p.), peritoneum opened and left testis was removed for further investigations (Minutoli *et al*., 2005).

Experimental procedure

Twenty four male Wistar rats were randomly divided into four experimental groups (n = 6) as follows:

The Sham group (A): no medication was employed; animals were exposed to midline laparotomy without clamping the IR. The IR group (B): rats were exposed to midline laparotomy with clamping of the 1 h ischemia and 24 h of reperfusion period, without pre-medication. IR + Tramadol (20 mg/kg). Group (C): 20 mg/kg tramadol was orally administered for 1 week prior and then the animals were exposed to midline laparotomy with clamping of the 1 h ischemia followed by 24 h of reperfusion. IR + Tramadol (40 mg/kg). Group (D): 40 mg/kg of tramadol for 1 week was orally administrated. Then, animals were exposed to midline laparotomy with clamping of the 1 h of ischemia and 24 h of reperfusion. In all experimental groups, animals were subjected to testicular ischemia followed reperfusion in the left testis. At the end 24 h, rats were euthanized, peritoneum opened and testes were taken out for histopathological investigations and semen MDA, SOD, GPx and TAS levels.

Tissue processing

Testis tissue samples from the experimental rats were fixed at 10% buffered formalin solution and processed for paraffin sectioning. Tissue section about 5µm thickness were taken and stained with hematoxylin and eosin (H & E; Wei *et al*., 2011). The testis sections were graded numerically to assess the degree of histological changes associated with seminiferous tubule injury as previously described by Johnsen *et al*. (1970) as bellow:

- 10: complete spermatogenesis and perfect tubules
- 9 : many spermatozoa present but disorganized spermatogenesis
- 8: only a few spermatozoa present
- 7: no spermatozoa but many spermatids present;
- 6: only a few spermatids present
- 5: no spermatozoa or spermatids present but many spermatocytes present
- 4 : only a few spermatocytes present
- 3 : only spermatogonia present
- 2: no germ cells present
- 1: neither germ cells nor Sertoli cells present

Measurement of SOD, MDA and GPx

At the end 24 h, semen samples were collected from the cauda of epididymis and homogenized in 10% (W/V) ice-cold buffer (0.1 M phosphate buffer, pH 7.4 + 150 mM KCl; Ghiasi Ghalehkandi *et al*., 2015). The homogenate was centrifuged at 9000 rpm for 20 min to obtain a supernatant, which was used for SOD, MDA, GPx and TAS estimations (Sharma *et al*., 2012). The role of SOD is to accelerate the dismutation of the toxic superoxide radical (O_2), produced during the 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 detectable at 505 nm (Woolliams *et al*., 1983). The MDA is a standard to determine free radical

damage. The MDA was formed as an end product of lipid peroxidation and treated with thiobarbituric acid (TBA) to produce a colored product that was measured at 532 nm (Placer *et al*., 1966). The GPx catalyzes the oxidation of glutathione and in the presence of glutathione reductase and NADPH, oxide glutathione is converted to the reduced form by changes in oxidation of NADPH to $NADP^{+}$. The GPx level was measured in absorbance of 340 nm (Paglia and Valentine, 1967). The TAS detecting kit was obtained from Randox (Randox Laboratories Ltd., Crumlin, Antrim, United Kingdom; Cat. no. NX2332). Antioxidants added to samples lead suppression in color production measured at 600 nm (Miller *et al*., 1993). The relationship between ROS, SOD, MDA, GPx TAS, PUAFAs on sperm/spermatogenesis is provided in Fig.1.

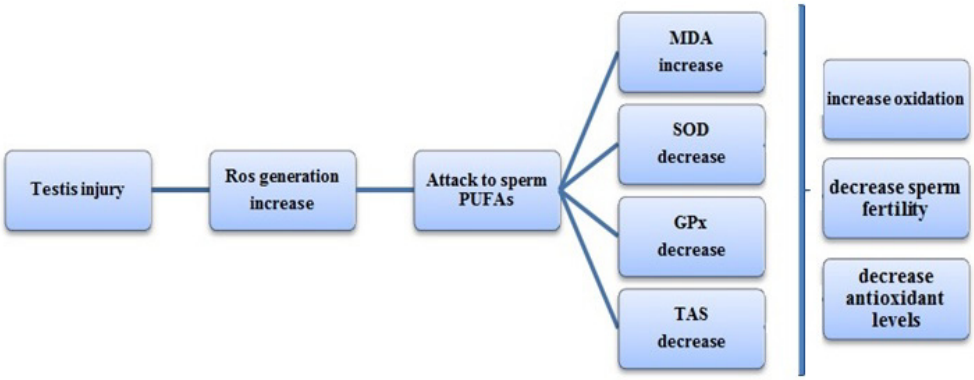

Figure 1. Relationship between ROS, SOD, MDA, GPx TAS, PUAFAs on sperm/spermatogenesis.

Statistical analysis

Data were prepared in excel, the parametric data analyzed with one way analysis of variance (ANOVA) using SPSS 16.0 for Windows (SPSS, Inc., Chicago, IL, USA). Data were expressed as mean values ± standard error of mean (SEM). For treatment showing a main effect by ANOVA, means compared using Duncan Multiple Range Test (Duncan, 1957). P values of <0.05 were considered to denote significant differences between groups.

Results

Result for a score of histological changes associated with seminiferous tubules injury based on Johnsen (1970) is presented in Fig. 2. Also, the effect of IR and administration of tramadol on testis histopathology after testicular IR injury is presented in Fig. 3-6. Finally, the effect of tramadol on semen MDA, SOD, GPx and TAS levels after testicular IR injury is shown in Table 1 and Fig. 7-10. As seen, lowest testis damage grade was observed in IR group compared to the sham group ($P < 0.05$). There was significant difference on testis damage score in rat treated with different levels of tramadol (20 *vs*. 40 mg/kg; $P < 0.05$; Fig. 2).

According to the results, sham group had normal seminiferous tubules and spermatogenesis with spermatocytes, Sertoli cells and spermatozoa in rat ($P > 0.05$; Fig. 3). Also, 1 h of testicular ischemia followed by 24 h of reperfusion injury showed degenerated seminiferous tubules and loss of spermatogenesis, seminiferous tubules with few spermatocytes in degenerated tubules compared to sham group in rat ($P < 0.05$; Fig. 4).

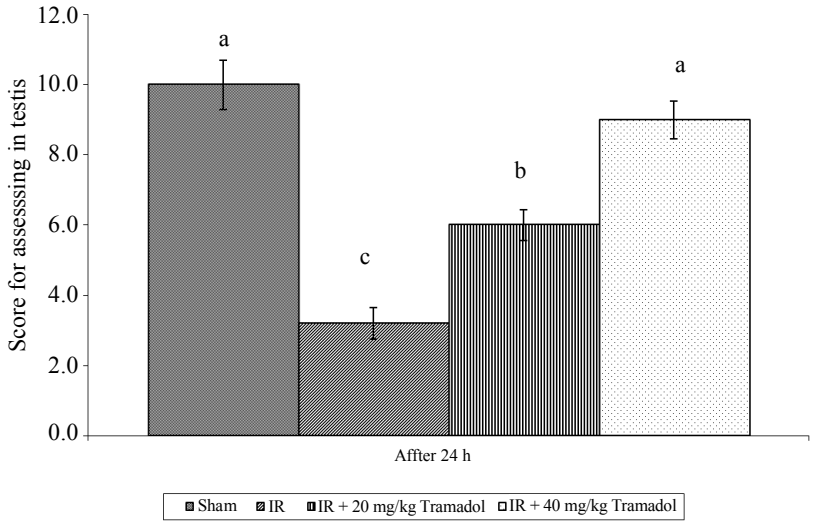

Figure 2. Results for score of histological changes associated with seminiferous tubules injury in unilateral IR injury in rat. Asterisks indicate significant difference on testis damage score between groups compared to varicocele group ($P < 0.05$). Different letters$^{(a,bc)}$ indicate significant differences between treatments ($P < 0.05$).

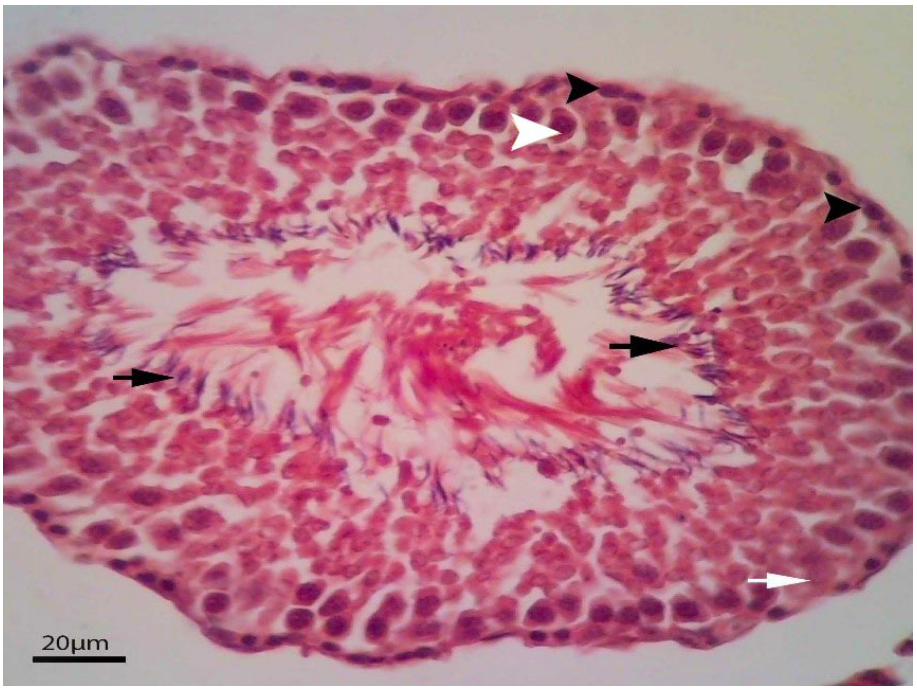

Figure 3. Testis section of sham group rats showing normal seminiferous tubules with many spermatozoa (black arrow), spermatogonium (black arrowhead), Sertoli cell (white arrow) and spermatocyte (white arrowhead). Hematoxylin and Eosin staining.

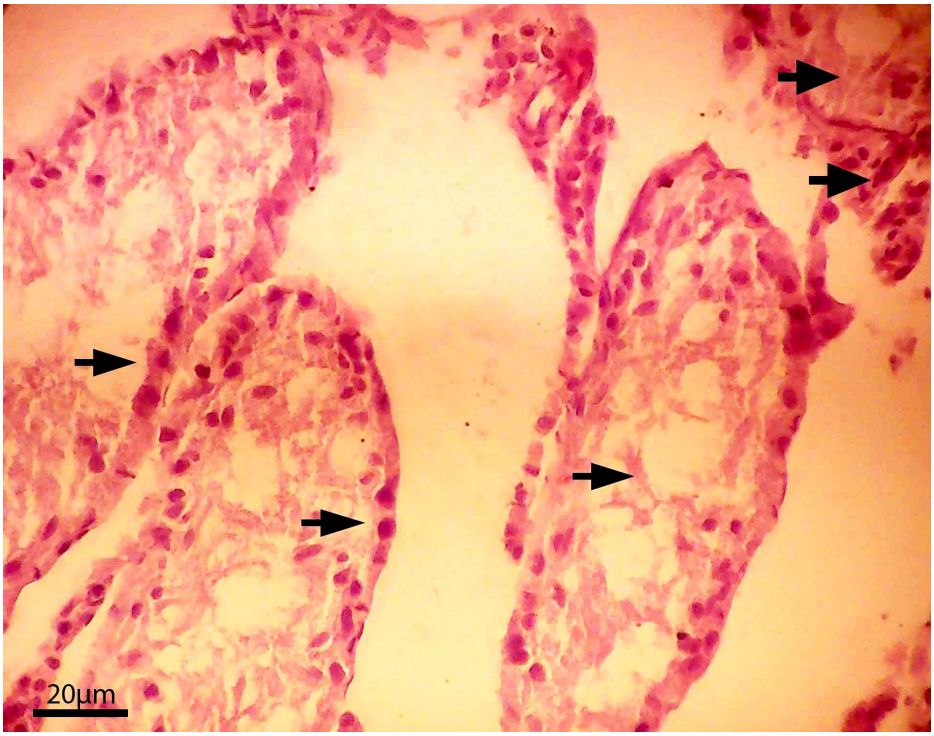

Figure 4. Testis section of testicular ischemia (1 h) followed by reperfusion (24 h) injury rats (without pre-treatment) showing degenerated seminiferous tubules (arrow) and loss of spermatogenesis. Hematoxylin and Eosin staining.

As seen in Fig. 5, administration of tramadol (20 mg/kg) for 1 week was not able to minimize testicular-induced IR injury compared to the sham group ($P > 0.05$). Also, administration of 40 mg/kg of tramadol (Fig. 6) significantly minimized testicular IR injury.

As seen in Table 1 and Fig. 7, MDA levels

significantly increased in IR rat compared to control group ($P < 0.05$). Also, the elevated levels of MDA significantly decreased in tramadol (40 mg/kg) treated rats compared to IR group ($P < 0.05$).

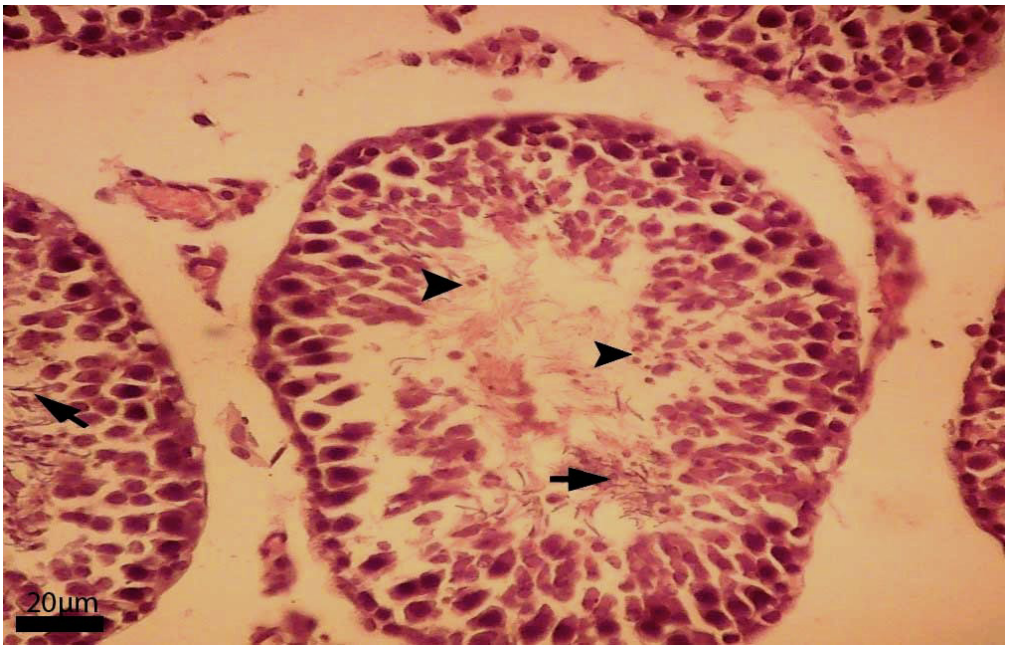

Figure 5. Testis section of testicular ischemia (1 h) followed by reperfusion (24 h) injury rats (pre-treated with 20 mg/kg of tramadol for 1 week) showing many spermatid (arrow head) and few spermatozoid (arrow). Hematoxylin and Eosin staining.

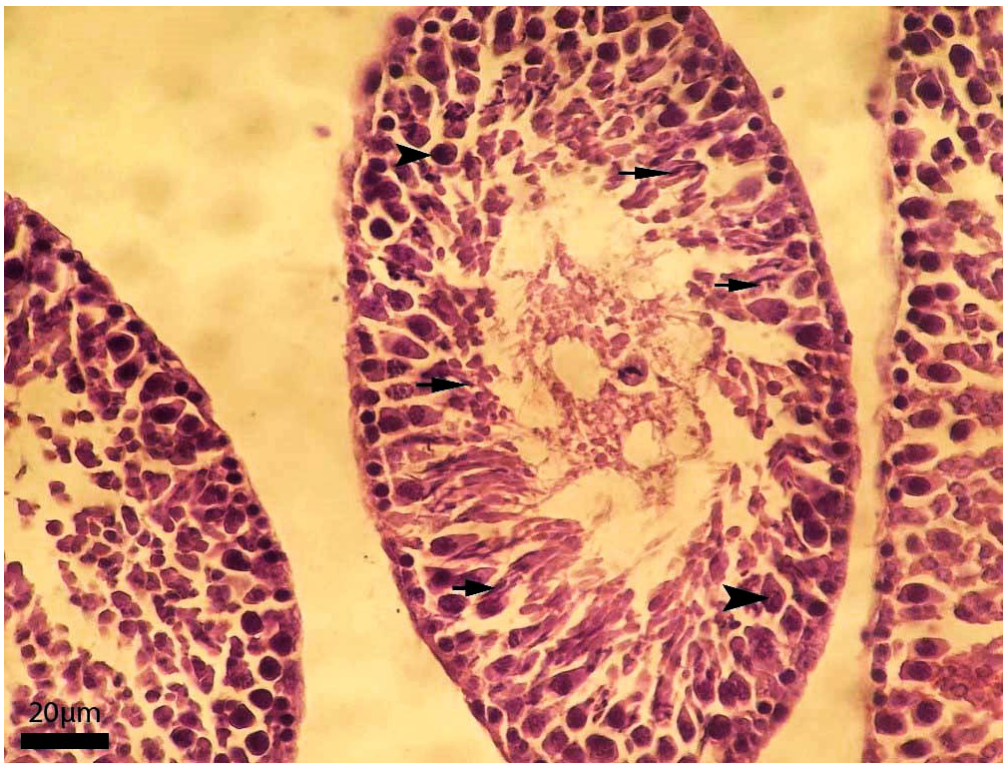

Figure 6. Testis section of testicular ischemia (1 h) followed by reperfusion (24 h) injury rats (pre-treated with 40 mg/kg of tramadol for 1 week) showing many normal spermatocyte (arrowhead) and spermatozoa (arrow). Hematoxylin and Eosin staining.

Table 1. Effect of tramadol administration on semen values of Malondialdehyde, Superoxide dismutase, Glutathione peroxidase and total antioxidant status in experimental testicular ischemia-reperfusion injury in rat.

Group	MDA (nmol/ml)	SOD (IU)	GPx (IU)	TAS (nmol/ml)
Sham	200.2 ± 6.01^{c}	199.20 ± 9.31^{a}	7189.50 ± 46.22^{a}	13.75 ± 0.91
IR	263.5 ± 4.60^{a}	129.57 ± 6.2^{c}	6516.6 ± 56.72^{c}	11.50 ± 0.50
tramadol (20 mg/kg) + IR	252.3 ± 5.20^{a}	136.16 ± 7.51^{c}	6824.46 ± 51.38^{c}	11.23 ± 0.27
tramadol (40 mg/kg) + IR	225.1 ± 5.77^{b}	174.16 ± 9.08^{b}	7050.02 ± 44.50^{b}	12.17 ± 0.49

Sham group: no medications, abdominal cavity opened without testicular ischemia-reperfusion injury. IR group: abdominal cavity was opened, testicular ischemia-reperfusion injury-induced without pre-medication. IR: ischemia-reperfusion, MDA: malondialdehyde, SOD: superoxide dismutase. GPx: glutathione peroxidase, TAS: total antioxidant status. ^{a,b,c} There are significant differences between groups with different superscripts in a column ($P < 0.05$).

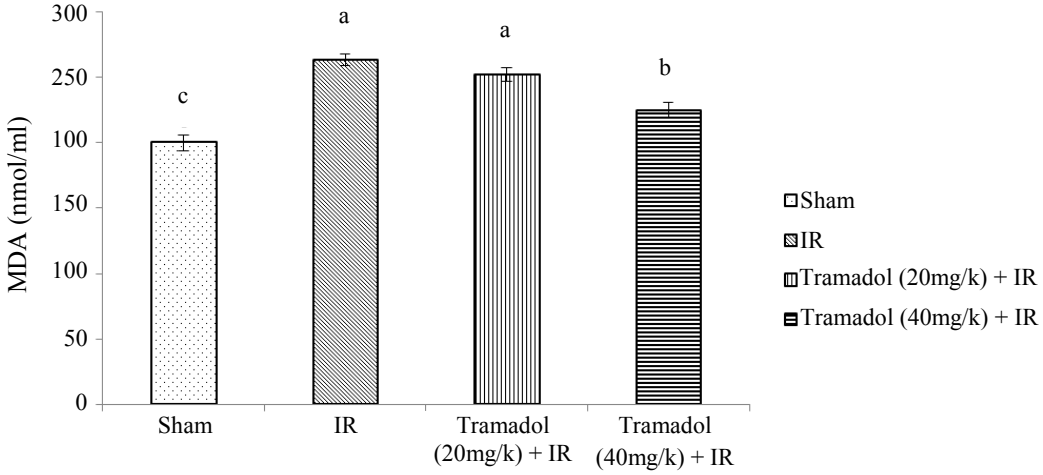

Figure 7. Effect of tramadol administration on semen values of Malondialdehyde (MDA) in experimental testicular ischemia-reperfusion injury in rat.

As seen in Table 1 and Fig. 8, SOD levels significantly decreased in IR rat compared to control group ($P < 0.05$). Also, the diminished levels of SOD significantly normalized in tramadol (40 mg/kg) treated rats compared to IR group ($P < 0.05$).

According to the result in Table 1 and Fig. 9, GPx levels significantly decreased in IR rat compared to control group ($P < 0.05$) while the diminished levels of GPx significantly increased in tramadol (40 mg/kg) received rats compared to IR group ($P < 0.05$).

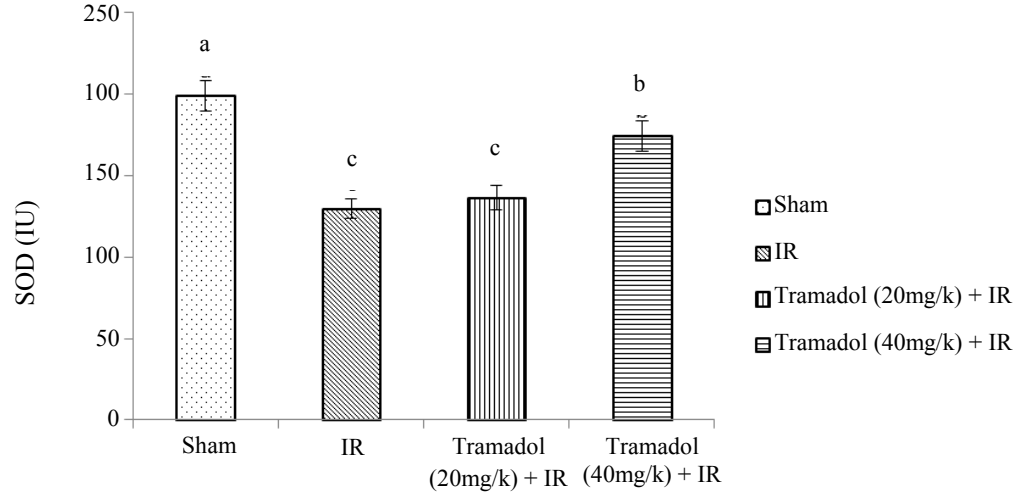

Figure 8. Effect of tramadol administration on semen values of Superoxide dismutase (SOD) in experimental testicular ischemia-reperfusion injury in rat.

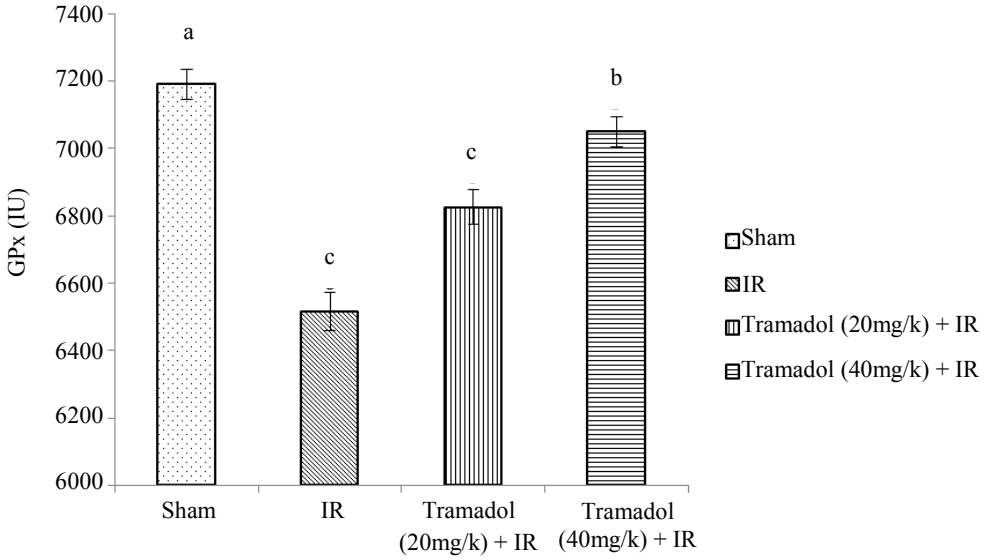

Figure 9. Effect of tramadol administration on semen values of Glutathione peroxidase (GPx) in experimental testicular ischemia-reperfusion injury in rat.

As presented in Fig. 10 and Table 1, there was no significant difference on TAS level among the tramadol-treated animals compared to sham and IR groups ($P > 0.05$).

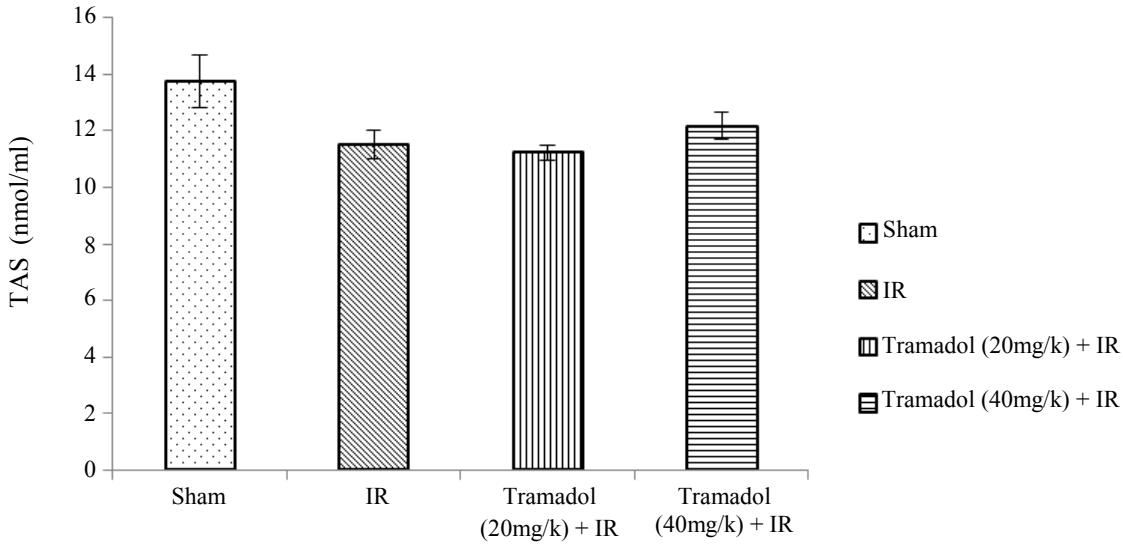

Figure 10. Effect of tramadol administration on semen values of total antioxidant status (TAS) in experimental testicular ischemia-reperfusion injury in rat.

Discussion

To the best of our knowledge, there are limited studies describing the role of tramadol on spermatozoa oxidative damage and testes pathology in testicular IR injury in rat. As observed in the study, testicular IR caused degeneration of the seminiferous tubules and loss of spermatogenesis and seminiferous tubules with few spermatocytes in degenerated tubules in rat. Testicular torsion results in infertility and testicular damage. Based on the reports the minimum time for testicular damage after experimental testicular torsion in the rat is 1 h (Koksal *et al*., 2012). Then, 1 h ischemia followed by 24 h reperfusion to determine property of tramadol as fast active medicine against testicular IR injury was used in present study.

Based on histological features observed in the present study, administration of 40 mg/kg of tramadol for 1 week prior to the initiation of the experiment protects testicular against IR injury but the level of 20 mg/kg had no effect. It is reported that high levels of tramadol (>40 mg/kg) has a toxic effect in male rats (El-Gaafarawi, 2006). So in this study, we used an effective dose of tramadol to investigate its effect as a curative medicine against testicular IR injury. Testicular torsion leads to ischemia and reperfusion with detorsion of the twisted testicle, which lead to morphological damage to testicular tissue. Additionally, post-ischemic reperfusion amplifies further tissue damage and apoptosis (Tuglu *et al*., 2015).

Testicular lesions were characterized by decrease in testicular weight, mean seminiferous tubular diameter, number of germ-cell layers and mean testicular biopsy score (Yurtçu *et al*., 2008, 2009; Wei *et al*., 2011). Despite several progress is done during the past decade in this area, IR injury remains a clinically challenging problem (Parlaktas *et al*., 2014). The present study showed unilateral testicular IR increased testicular MDA level (an indicator of ROS content; Table 1) and caused a significant decrease in spermatogenesis, seminiferous tubules with few spermatocytes in degenerated tubules (Fig. 4). Also, SOD and GPx levels diminished while TAS remained intact.

In present study, 1 h ischemia followed by reperfusion significantly increased MDA level while decreased SOD and GPx activity. Seminal plasma is endowed with frequent enzymatic antioxidants includes MDA, SOD and GPx (Ghiasi Ghalehkandi *et al*., 2015). The pathophysiological mechanisms involved in organ damage caused by testicular torsion might link strictly to the ischemia during torsion and subsequent events after reperfusion. In fact, during the early stage of IR injury, massive release of ROS after reperfusion followed by endothelial dysfunction or neutrophil infiltration triggers the oxidative damage (Tüfek *et al*., 2013). In this regard, Wei *et al*., (2011) reported 2 h of unilateral testicular torsion followed by detorsion changes in MDA, SOD and CAT activities. Sperm membranes contain high levels of polyunsaturated fatty acids (PUFAs) in spermatozoa which is susceptible to be attacked by ROS (Hadwan *et al*., 2014).

The generation of oxygen-derived free radicals is responsible for IR in various organs (Koksal *et al*., 2012). ROS lead to produce hydrogen peroxide and free radicals. These free radicals have unpaired electrons in their outer orbits which can impress their effect via oxidative stress. Oxidative stress, resulting in an imbalance between the production of free oxygen radicals and antioxidant capacity, leads to damage on cell (Zhang *et al*., 2012). The ROS severely produce from of abnormal spermatozoa which decrease antioxidant defenses in the seminal plasma (Agarwal *et al*., 2009).

In recent years, several antioxidant agents have been used to prevent testicular IR tissue damage. For instance, Ashrafzadeh Takhtfooladi *et al*. (2015b) reported that an intravenous injection of 20 mg/kg of tramadol normalized MDA, SOD and CAT levels in myocardial IR in rat. Furthermore, Nagakannan *et al*. (2012) reported pretreatment of male Wistar rat with tramadol (20 mg/kg of for 4 days) had the neuroprotective effect against transient forebrain ischemia. Also, Bilir *et al*. (2007) reported infusion of tramadol at the concentration of 1×10^4 M/L for 10 min diminished MDA level while increased SOD and GPx activity in myocardial IR injury rats. In our study, administration of tramadol (40 mg/kg) normalized the oxidative stress induced enzyme levels which was similar to the previous observations (Bilir *et al*., 2007; Nagakannan *et al*., 2012; Ashrafzadeh Takhtfooladi *et al*., 2015b).

MDA is the end product of lipid peroxidation, where the elevation in testicle MDA levels is a marker for extent of oxidative stress and leads to infertility (Hsieh *et al*., 2006). The elevated level of MDA is an index of the extent of lipid peroxidation and oxidative stress. As observed, levels of MDA decreased in the group receiving tramadol compared with the IR group supports the hypothesis tramadol might reduce oxidative stress by scavenging peroxyl radicals. So, low levels of ROS are critical for normal spermatogenesis and fertility (Agarwal *et al*., 2009). The SOD is a fundamental part of the cellular antioxidant defense system (Ghiasi Ghalehkandi, 2015). It is the first defense line against oxidative stress with dismutation of superoxide anion radicals to H_2O_2 (Asadpour *et al*., 2013). GPx is an enzyme family with peroxidase activity; its activity depends on reducing levels of glutathione, glutathione transferase, and glutathione reductase. GPx impresses its role by protecting sperm against peroxidative damage (Hsieh *et al*., 2006). A correlation exists between GPx levels and asthenozoospermia in which its activity develops an essential role in the cellular defense against free radicals (Ghiasi Ghalehkandi, 2015). Superoxide dismutase converts superoxide anion to hydrogen peroxide (Hsieh *et al*., 2006).

Based on the literature, scarce investigations have been done on role of tramadol against testicular IR injury. In conclusion, the findings of the present study have demonstrated that the testicular damage occurs following IR. It is well documented repairing the harmful effects of oxidative stress on reproductive tissues with antioxidant agents is the first line of treatment (Koksal *et al*., 2012). As observed tramadol might be a potent agent in preventing testicular IR injury by normalize the oxidative enzyme levels (MDA, SOD, GPX and TAS levels) in rat. This information can be useful as base data for further investigations as an antioxidant agent in human testicular torsion.

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