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Minoxidil reduces testicular damage in experimental testicular ischemia/reperfusion injury

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We investigated the effects of K_{ATP} channel opener minoxidil on oxidant stress, antioxidant defense and histopathological damage in testicular torsion-induced rats. A total of 21 male Wistar-Albino rats were used. Group 1 (the sham-control): the left testis and spermatic cord were only released with a scrotal incision. Group 2 (I/R-untreated): the left testis and spermatic cord was rotated clockwise by 720 degrees for 60 minutes and detorsioned for 60 minutes. Group 3 (I/ R- minoxidil treated): Testicular torsion was created as in the group 2 rats and 0.3 mg / kg minoxidil was injected. The testes were extracted for biochemical and histopathological examination.Minoxidil treatment showed a significant decrease in MDA and MPO levels. In the group 2, there was a decrease in TAS levels and an increase in TOS levels. Minoxidil treatment resulted in an improvement of these values in the testicular tissues. There was an improvement in the testicular injury score with minoxidil treatment. The group 2 rats had grade III and grade IV lesions. The treatment with minoxidil showed lower histopathologic features in the testicular tissues. Minoxidil may play a protective role in reducing damage caused by reperfusion of the ischemic testicular tissues.

Keywords: Testes torsion, ischemia reperfusion injury, oxidative stress, minoxidil, rats

INTRODUCTION

Testicular torsion is a urological emergency, with an estimated incidence in one to 160 men under the age of 25 every year (Barada et al., 1989; Wampler and Llanes 2010). Although urgent detorsion intervention is necessary for testicular tissue to survive, damage during ischemia-reperfusion (I/R) may lead to germ cell apoptosis, impaired spermatogenesis, and even male infertility (Zhou et al., 2019; Lee et al., 2018). Anoxia, activation of leukocytes, release of inflammatory mediators, and release of reactive oxygen species (ROS), such as superoxide radical and hydrogen peroxide, are some of the proposed mechanisms of I/R injury (Willerson 1997; Pompermayer et al., 2007). Among these, ROS have been shown to be a main cause of reperfusion injury (Das and Sarkar 2005). ROS increase membrane permeability or disruption of membrane integrity—and, consequently, DNA damage—through lipid peroxidation (Yildirim et al., 2018).

Minoxidil (2,4-diamino-6-piperidinylpyridine 3-oxide) directly affects the smooth muscles of vessel walls by opening ATP-sensitive potassium (K_{ATP}) channels. Therefore, it is known as a potent peripheral vasodilator agent (Bittencourt Rde et al., 2005; Takatani et al., 2004; Meisheri et al., 1988).

Currently, minoxidil is widely used to treat baldness because it leads to proliferation of hair follicle epithelial cells through vasodilation in the scalp (Bittencourt Rde et al., 2005; Savin and Atton 1993). Minoxidil has also been a research topic in some experimental studies, such as thos concerning ischemia-induced apoptosis, ischemic cutaneous flaps, and myocardial ischemia and reperfusion (Bittencourt Rde et al., 2005; Takatani et al., 2004; Baczkó et al., 1997; Yamamoto et al., 2002).

Therefore, this study investigated, for the first time, the effects of the K_{ATP} channel opener minoxidil on oxidant stress, antioxidant defenses, and histopathological damage in the testicles during reperfusion.

MATERIALS AND METHODS

This experimental study was approved by the Bolu Abant Izzet Baysal University Animal Care Local Ethics Committee (HAYEK) (Decision no: 2018/10). The 21 male Wistar Albino rats used in this study (3months old, weighing 240 g to 280 g) were obtained from the HAYEK animal care department. The rats received a standard diet, tap water, and libitum in the laboratory and were kept at normal room temperature (22ºC).

Rats were anesthetized with intramuscular injections of a mixture of ketamine (50mg/ml) and 1 ml of xylasine (0.2 ml/100g of weight). Half of the initial dose was used as needed during the procedure

The rats were divided into three groups of seven animals. The scrotal area was then stained with 10% povidone iodine solution for antisepsis. In the Group 1 rats (the sham-control, n = 7), the left testis and spermatic cord were released through a scrotal incision. The testis was placed in the scrotum without creating testicular torsion. In the Group 2 rats (I/R-untreated, n=7), after release of the left testis and spermatic cord, the testis cord was rotated clockwise by 720° and was fixed to the inner surface of the scrotum with 6/0 propylene. The Group 2 rats underwent torsion for 60 m and detorsion for 60 m. The rats were not given any treatment. In the Group 3 rats (I/R-Minoxidil treated, n=7), testicular torsion was created as in the Group 2 rats. 0.3 mg/kg of minoxidil (Sigma-Aldrich) was injected intravenously over a period of 30 s into the left femoral vein, at a volume of 0.1 ml/kg. After 60 m of torsion, reperfusion was achieved with 60 m of detorsion.

At 60 m post-detorsion, the testes were numerated and fixed in 10% neutral formaldehyde solution for biochemical and histopathologic examination.

Then, the rats in each group were decapitated under anesthesia via guillotine and the experiment was finalized.

Measurements of malondialdehyde, myeloperoxidase activities, and lipid peroxidation

The testis tissue samples were washed with phosphate buffered saline and were stored at -80°C until the day of biochemical analysis. When analysis began, the homogenate was centrifuged at 4°C and 1,600 g for 10 m, and the supernatant was removed. Myeloperoxidase (MPO) was measured using commercially available, enzyme-linked, immunosorbent assay kits, according to the manufacturer's instructions (ELISA Kit for MPO, Cloud-Clone Corp., USA). Malondialdehyde (MDA) was measured using commercially available, colorimetric assay kits, according to the manufacturer's instructions (Thiobarbituric Acid Reactive Substances (TBARS, TCA Method) Assay Kit, Cayman Chemical, USA). Bicinchoninic acid (BCA) protein assay was used for the quantitation of tissue total protein (Thermo Fisher Scientific Inc., Rockford, IL, USA).

Tissue total antioxidant status (TAS) and total oxidant status (TOS) levels

To measure TAS and TOS levels in the testicular tissues, a Beckman Coulter AU680 analyzer with commercial reagents (Beckman Coulter) was used (Rel Diagnostic Assay, Gaziantep, Turkey).

Histologic grading

The orchiectomized testicular tissues were immediately placed into a 10% formalin solution. The tissue specimens, taken into paraffin blocks, were sectioned at 5 µm and stained with hematoxylene and eosine (H&E). The sections were blindly examined under light microscope (Olympus BH-2, Olympus Corporation, Tokyo, Japan) by two investigators. In evaluating the histological parameters, the classification of Cosentino et al. (1985) was used. Grade I showed normal testicular architecture, with an orderly arrangement of germinal cells. Grade II injury demonstrated less orderly, noncohesive germinal cells and closely packed seminiferous tubules. Grade III injury exhibited disordered, sloughed germinal cells, with shrunken, pyknotic nuclei and less distinct seminiferous tubule borders. Grade IV injury defined seminiferous tubules that were

closely packed, with coagulative necrosis of the germinal cells.

Statistical analyses

All values were expressed as mean ± standard deviation. The significance of the data obtained from oxidative stress-associated parameters was evaluated using analysis of variance (ANOVA). Differences between means were analyzed via post-analysis test after ANOVA (Tukey's b test). The Mann-Whitney U test and Kruskal-Wallis test were also used to compare statistical analysis of the histologic data. P values of < .05 were considered significant.

RESULTS

The MDA, MPO, TAS, and TOS values measured in the testicular tissues are shown in Table 1. Testis torsion caused a significant increase in MDA and MPO levels in the I-R/ untreated group compared to the other groups (P < .0001, P < .0001, respectively). Minoxidil treatment showed a significant decrease in MDA and MPO levels in the testicular tissues (P < .0001, P < .0001, respectively). In the I-R/untreated group, TAS levels decreased, and in TOS levels increased, in the testicular tissues (P < .0001, P < .0001, respectively). In contrast, minoxidil treatment resulted in an improvement of these values in the testicular tissues (P < .0001, P < .05, respectively).

The testicular injury score was increased in the I-R/untreated group compared to the sham group (P < .0001). In contrast, the testicular injury score improved with minoxidil treatment compared to the I-R/untreated group (P < .0001) (Figure 1).

Table 1:Oxidative stress associated parameters in the testes tissues of the groups.

Groups	MDA nmol/g protein	MPO ng/g protein	TAS mmol/L	TOS µmol/L
G1 (N=7)	3,23±0,49	14,84±2,42	1,31±0,04	7,01±1,20
G2 (N=7)	16,72±2,62*	26,45±2,98*	1,01±0,11¶	22,06±3,75*
G3 (N=7)	7,86±1,12*†	20,50±0,70*†	1,57±0,09▲	10,43±1,54*†

Values are mean ± SD. G1: Sham-control, G2: I/R-untreated, G3: I/R-Minoxidil treated, MDA: Malondialdehyde, MPO: Myeloperoxidase, TAS: Tissue total antioxidant status, TOS: Total oxidant status.
*P <.05, compared with group sham-control. †P <.05, compared with I-R/untreated group.
¶P <.05, compared with group sham-control. ▲P <.05, compared with group sham-control and I-R/untreated group.

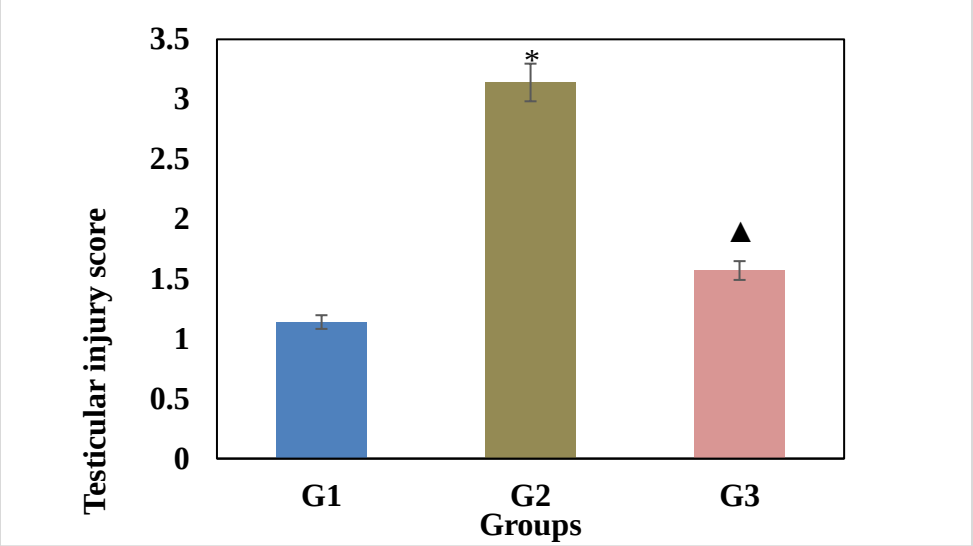

Figure 1. Comparative testicular injury score measurements of the groups. Values are mean ±SD. G1: Sham-control, G2: I/R-untreated, G3: I/R-Minoxidil treated.
*P <.05, compared with group sham. ▲P <.05, compared with I-R/untreated group.

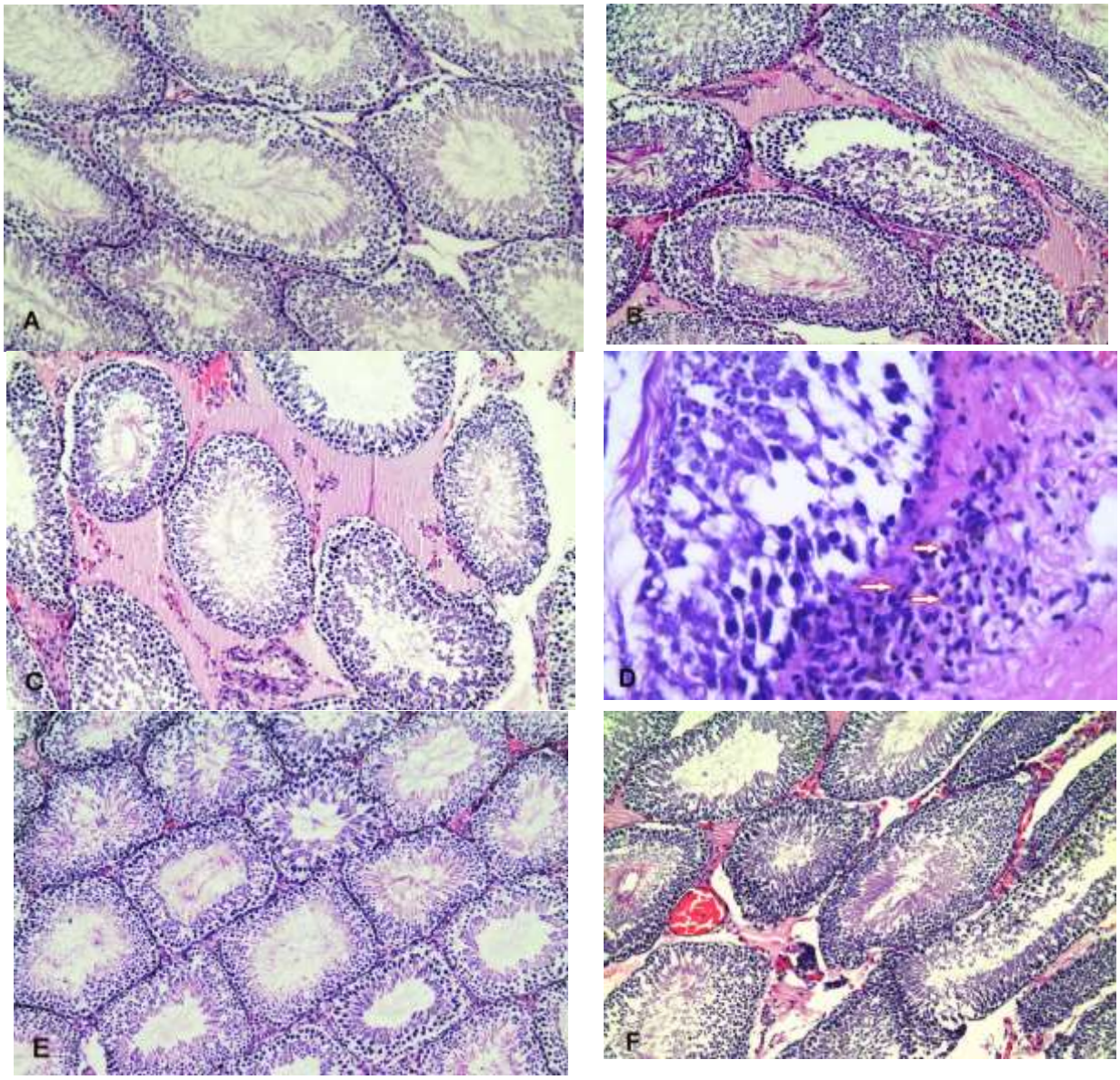

Figure 2: Photomicrographic view of the testicular tissues. (A) The sham-control group showed normal seminiferous tubule morphology and the orderly arrangement of germinal cells. (H&E, x100). (B, C) The lesions in I-R / untreated rats ranged from grade III to grade IV, including testicular edema, congestion, bleeding and necrosis of the germinal cells. (H&E, x100). (D) In the I-R/untreated group rats; note the infiltrating leukocytes (arrows) (H&E, x200). (E, F) In the I-R/minoxidil-treated group, most of the testicular samples showed grade I-II injury (H&E, x100).

The sham group rats had normal testicular architecture with an orderly arrangement of germinal cells (Figure 2A). The rats with testicular torsion had Grade III and Grade IV lesions, such as edema, congestion, leukocyte infiltration, bleeding between the seminiferous tubules, and necrosis of germinal cells (Figure 2B, C and D). However, minoxidil-treated the rats showed lower Grade I and II histopathological findings in testicular tissues (Figure 2E, F).

DISCUSSION

It is known that testicular torsion causes testicular damage, apoptosis, and even infertility,

as a result of I/R. Oxygen-free radicals formed during testicular I/R are one of the most important pathological factors accused for this damage. Excessive accumulation of oxygen-free radicals leads to lipid peroxidation and deterioration of membrane permeability and integrity, resulting in DNA damage and testicular germ cell apoptosis (Ikeda et al., 1999; Ford 2004; Filho et al., 2004). In addition, local and systemic inflammatory response affects the pathophysiology of I/R injury. Thus, an increase in microvascular permeability leads to leukocyte migration and platelet-leukocyte aggregation (Elshaari et al., 2011; Wei et al., 2011). When the I/R process is not intervened with, testicular damage is further enhanced under the influence of these pathophysiological events. In experimental studies, many drugs and chemical substances for preventing tissue damage from testicular torsion have been researched. However, strong results from experimental studies could not enter clinical practice due to the serious side effects of the drugs (Zhou et al., 2019; Lee et al., 2018; Yildirim et al., 2005). In the present experimental study, the protective effects of minoxildil in the testicular I/R model were investigated for the first time. The results showed that minoxildil effectively prevents testicular I/R damage via its strong vasodilator effect and its antioxidant and antinflammatory properties.

Although side effects such as ventricular arrhythmia and hypotension limit the clinical use of K_{ATP} channel openers, minoxidil is a potent K_{ATP} channel opener and has a variety of effects, from vasodilation to promoting hair growth (Toshiaki et al., 2004; DeVillez 1990). Therefore, these compounds are effective in protecting cells from I/R damage and deserve further investigation (Toshiaki et al., 2004). K_{ATP} activity has been detected both at the sarcolemmal membrane level (sarcK_{ATP}) and at the mitochondrial level (mitoK_{ATP}), and it is a common target of potassium channel openers (KCOs) (Mannhold 2004; Campbell et al., 2003). K_{ATP} channels are inhibited by physiological intracellular ATP levels and are found in their own specific phenotypes in different tissues, such as the central nervous system, the cardiovascular system, the lungs, the pancreas, and the bladder (Mannhold 2004; Campbell et al., 2003). Some experimental studies investigating the clinical use of KCOs have identified many effects of these chemicals, including their acting as bronchodilators, vasodilators, and bladder relaxants (Jahangir et al., 2001). ATP-sensitive K+ channel (K_{ATP}) opener minoxidil may be beneficial because of several mechanisms as indicated by Garlid (1996): “The existence in mitochondria of separate, highly regulated pathways for K+ efflux and influx strongly implies that mitochondrial volume is subject to regulation in vivo. Volume, in turn has been shown to regulate activity of the electron transport chain. Mitochondrial K_{ATP} channels by controlling the mitochondrial K+ cycle are thought to be involved with mitochondrial volume control and cellular bioenergetics”. Bittencourt et al. (2005) evaluated the effect of minoxidil in preventing necrosis in potential ischemic flaps in rats. They found that minoxidil sulfate was effective in preventing necrosis in ischemic flaps, but, after necrosis, there was no difference in survival between the experimental and control groups. In an acute cardiac ischemia model in rabbits, Das et al. (2005) suggested that intervention with minoxidil, the selective activator of mitochondrial K_{ATP} channels, had cardioprotective effects. Takatani et al. (2004) evaluated the effects of minoxidil on ischemia-induced necrosis and apoptosis using a simulated ischemia cardiomyocyte model. Their results showed that minoxidil renders cells resistant to ischemia-induced necrosis and apoptosis due to its ability to open mitochondrial K_{ATP} channels. As mentioned above, I/R leads to ROS formation and subsequent lipid peroxidation. Byproducts, such as aldehydes, secondary to lipid peroxidation, exacerbate oxidative damage. These molecules interact with biomolecules, such as nucleic acids and proteins, causing irreversible damage to the main mechanisms of cell function (Rio D et al., 2005). MDA is the main, and most studied, product of polyunsaturated fatty acid peroxidation and is used as an indicator of oxidative stress in cells and tissues (Ozer et al., 2005). Testicular torsion causes a significant increase in neutrophil adhesion to the testicular venous endothelium. In the subsequent process, tissue damage occurs in various forms, such as the secretion of proteolytic enzymes, like elastase, from cytoplasmic granules, the production of free radicals, the physical disruption of microcirculation, and the prolongation of ischemia. MPO activity is known as an index of tissue-associated neutrophil accumulation (Lysiak et al., 2001; Wang et al., 2008). In the present study, minoxidil treatment showed a significant decrease in MDA levels in the testicular tissues. In addition, minoxidil treatment provided significant TAS and TOS improvement in ischemic testicular tissues. It has been shown in the present study that MPO activity, an index of tissue-associated neutrophil accumulation, increased in testis tissue 60 m after reperfusion and that the increase in

MPO activity was significantly inhibited by treatment with minoxidil. Tissues of rats treated with minoxidil showed a lower testicular injury score and lower histopathological features, such as Grade I and II.

CONCLUSION

As a result, due to above properties of minoxidil, its administration inhibited free radical production. Consequently, minoxidil decreased MDA and TOS production and increased TAS content. These positive results in minoxidil groups were also associated with improved histopathological appearnce. Low-dose intravenous minoxidil injection significantly improved testicular damage during reperfusion of ischemic testicular tissues.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

AUTHOR CONTRIBUTIONS

HO designed and performed the experiments and also wrote the manuscript. SED, OMY, AC, NC and HO performed animal treatments, testicular torsion experiments, tissue collection, and data analysis. HO and AC designed experiments and reviewed the manuscript. All authors read and approved the final version.

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