Beneficial effect of pentoxifylline into the testis of rats in an experimental model of unilateral hindlimb ischemia/reperfusion injury

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ABSTRACT

Objective: The objective of the present study was to investigate the role of pentoxifylline (PTX) on remote testicular injury caused by unilateral hind limb ischemia/reperfusion of rats.

Materials and Methods: Twenty healthy male Wistar rats were allocated randomly into two groups: ischemia/reperfusion (IR group) and ischemia/reperfusion + pentoxifylline (IR+PTX group). Ischemia was induced by placement of a rubber tourniquet at the greater trochanter for 2h. Rats in IR+PTX group received PTX (40 mg/kg IP) before the reperfusion period. At 24h after reperfusion, testes were removed and levels of superoxide dismutase (SOD), malondialdehyde (MDA), catalase (CAT) and myeloperoxidase (MPO) activity were determined in testicular tissues. Three rats of each group were used for wet/dry weight ratio measurement. Testicular tissues were also examined histopathologically under light microscopy.

Results: Activities of SOD and CAT in testicular tissues were decreased by ischemia/reperfusion (P<0.05). Significantly increased MDA levels in testicular tissues were decreased by PTX treatment (P<0.05). MPO activity in testicular tissues in the IR group was significantly higher than in the IR+PTX group (P<0.05). The wet/dry weight ratio of testicular tissues in the IR group was significantly higher than in the IR+PTX group (P<0.05). Histopathologically, there was a statistically significant difference between two groups (P<0.05).

Conclusions: According to histological and biochemical findings, we conclude that PTX has preventive effects in the testicular injury induced by hind limb ischemia/reperfusion.

INTRODUCTION

Hind limb ischemia/reperfusion injury may occur clinically after a release of tourniquets during orthopedic surgery, or extrication of a trauma victim who is compressed with a heavy weight for a prolonged period (crush syndrome). Several reports have indicated that the pathophysiology of the injury varies according to the duration and grade of the ischemia (1-3). It is well recognized that sustained ischemia can induce cell death and tissue necrosis, which are mainly caused by energy insufficiency. Many studies have shown that tissue injury in many organs occurs not only during the ischemic period but also in the reperfusion period. Skeletal muscle ischemia/reperfusion

is associated with a systemic inflammatory response and determines the effect on remote organs (liver, lung, kidney, myocardium and testis) structure and function (4-9). The development of remote organ dysfunction was observed only following reperfusion, which implies that humoral and/or cellular mediators produced locally in the limb were responsible for mediating remote organ injury (10-12).

Pentoxifylline (PTX) is a methylxanthine derivative with multiple hemorheologic properties. PTX through effects of increasing intracellular cyclic AMP on red blood cells improve the oxygen delivery to ischemic tissues and also increases the cyclic AMP on polymorph nuclear leukocytes and decreases oxygen free radical production (10, 13-16). In addition, PTX limit the inflammatory response with reduction in cellular activation, phagocytosis and endothelium adhesion. There are evidences that PTX also reduces the nitric oxide destruction (15). Recent studies have indicated that PTX improves ischemia/reperfusion injury in many organs (17-19). However, the effects of PTX on remote testicular injury caused by skeletal muscle ischemia/reperfusion are not clear. The objective of the present study was to investigate the role of PTX on remote testicular injury caused by unilateral hind limb ischemia/reperfusion of rats. For this purpose, the biochemical and pathological effects of skeletal muscle ischemia/reperfusion and PTX in testicular tissues of rats have been investigated. The results of our investigation would help to clarify the potential importance of the use of PTX in situations of oxidative damage. These findings may encourage the use of antioxidants to reduce remote organ injury after skeletal muscle ischemia/reperfusion.

MATERIALS AND METHODS

Animals

The study was conducted on 20 healthy male Wistar rats, 12-16 weeks old and weighing between 270-300g. All rats of this study were kept according to the norms of the Islamic Azad University College of Veterinary Medicine Tehran Iran laboratory of animal experimentations; this investigation was approved by the Committee of Ethics in Research with animals of Islamic Azad University. The study was designed so as to minimize the number of animals required for the experiments. The rats were housed in individual cages under temperature controlled standard conditions, 12h/12h light/dark cycle with free access to standard rodent food and filtrated tap water.

Experimental Design

The animals were allocated randomly into two experimental groups of ten rats each as follows: ischemia/reperfusion (IR group) and ischemia/reperfusion + pentoxifylline (IR+PTX group). Anesthesia was induced with a mixture of ketamine and xylazine (50 mg/kg and 10 mg/kg, respectively) via intramuscular injection. The level of anesthesia was continuously monitored by observing the respiratory patterns and toe pinch reflex. After the induction of anesthesia, fur was completely removed from left hind limbs with an electric shaver to facilitate the measurement of limb perfusion. Unilateral hind limb ischemia was induced by placing an orthodontic rubber band at the hip joint. Before using the tourniquet, 250 IU of heparin was administered via the jugular vein to prevent clotting. The animals were kept anesthetized throughout the duration of ischemic period with additional anesthetic doses as needed. Body temperature was maintained with a heating pad whilst the animals were under anesthesia. In IR+PTX group, pentoxifylline (40 mg/kg) was injected intraperitoneally immediately before reperfusion. Pentoxifylline tablets were dissolved in normal saline as explained in previous studies (20). The analgesic nalbuphine hydrochloride (2 mg/kg) was used via subcutaneous during observation time. After 24h of reperfusion, all animals were anesthetized again with the same mixture and a longitudinal incision of approximately 2cm was made to the scrotal raphe for bilateral orchiectomies (a procedure involving cord clamping and hemostasis). Three rats of each group were used for wet/dry weight ratio measurement. Right testes of seven rats were processed for histopathological examination and left testes were washed with normal saline and stored in a -80°C refrigerator for the evaluation of biochemical parameters. Then the rats were euthanized with an

overdose of intraperitoneal pentobarbital by injection (300mg/kg).

Preparation of Testicular Tissue Homogenates

The left testicular tissues were washed three times in cold normal saline solution (0.9%). Then, the tissues were homogenized in ice-cold Tris-HCl buffer solution, within a homogenizer for 2min at 11200×g. The homogenate was centrifuged at 3500×g for 60min and a supernatant was obtained. The levels of MPO were determined in the supernatant, and MDA levels were studied in the homogenate. For a further extraction procedure, the supernatant was extracted in ethanol/chloroform mixture (5/3 v/v). After a second centrifugation at 3500×g for 20min, the clear upper layer was taken and used for SOD activity determination (21).

BIOCHEMICAL ASSAYS

Measurement of SOD Activity

The principle of the SOD activity determination method was based on the inhibition of nitroblue tetrazolium reduction described by Sun et al. (22) and modified by Durak et al. (23). One unit of SOD was defined as the enzyme activity causing 50% inhibition in the nitroblue tetrazolium reduction rate. The SOD activity was expressed as units per mg tissue protein.

Measurement of MDA levels

The MDA levels in testicular tissues were analyzed by a method based on the reaction with thiobarbituric acid at 95ºC (24). In the thiobarbituric acid test reaction, MDA or MDA-like substances and thiobarbituric acid react together to produce a pink pigment with an absorption maximum of 532nm. The results were expressed as nanomol per gram wet tissue (nmoL/g tissue).

Measurement of CAT activity

CAT activity was determined according to Aebi's method (25). The principle of the assay is based on determination of the rate constant k (s^{-1}) of H_2O_2 decomposition at 240nm. Results were expressed as k (rate constant) per gram of protein.

Measurement of MPO activity

Testicular injury was quantified by measuring testicular MPO activity, the activity of infiltrated polymorphonuclear leukocytes, using a protocol modified from a previous report (26). MPO activity was determined after adding O--dianisidine dihydrochloride and hydrogen peroxide. The MPO activity was expressed as units per gram tissue.

Wet/dry weight assay

The wet/dry weight ratio, the tissue edema index, was measured to evaluate testicular injury. Briefly, freshly harvested testes were weighed, placed in an oven for 24h at 60°C and weighed again when dry (26). The wet/dry weight ratio was then calculated.

HISTOPATHOLOGICAL EVALUATION

The right extracted testes were immediately placed into 10% neutral formaldehyde solution. The tissue specimens were placed in paraffin blocks, sectioned at 5μm, and stained with hematoxylin and eosin (H&E) for light microscopic analysis. An experienced pathologist, who was blinded to the experiment and data, examined the samples histopathologically. The histological parameters were scored according to Cosentino et al. (27) classification as follows: Grade-I: Showed normal testicular architecture with an orderly, arrangement of germinal cells; Grade-II: Injury showed less orderly, non-cohesive 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 analysis

Analyses were performed by using commercial SPSS software for Windows version 18. Kruskal-Wallis test was used to compare the biochemical and histopathological parameters between groups. When Kruskal-Wallis test results were

significant, Bonferroni adjusted Mann-Whitney U test was used in the paired comparison. Data are shown as the mean±standard deviation with significance considered at 0.05.

RESULTS

The experimental procedure was well tolerated and no animal died during the experiment. One of the most interesting observations at the histopathological examination of the testicular tissues is the presence of sloughed germinal cells within the seminiferous tubules and disorganization in rat testes after the unilateral hind limb ischemia/reperfusion. The testicular injury score increased significantly in the IR group compared with the IR+PTX group (P<0.05) (Table-1). In the IR group, coagulative necrosis with loss of seminiferous tubule epithelium, edema and sloughed germinal cells were predominant features in sections (Figure-1). However, the rats in the IR+PTX group had essentially normal seminiferous tubule morphology (Figure-2).

Activities of SOD and CAT in testicular tissues were decreased by ischemia/reperfusion, but administration of PTX increased these levels (P<0.05) (Table-1). The tissue levels of MDA increased in the IR group in comparison to the IR+PTX group (Table-1). MPO activity in testicular tissues in the IR group was significantly higher than in the IR+PTX group (P<0.05) (Table-1). The wet/dry weight ratio of testicular tissues in the IR group was significantly higher than in the IR+PTX group (P<0.05) (Table-1).

DISCUSSION

The systemic inflammatory response syndrome is a consequence of many conditions, such as surgery, trauma, burn, shock, or bacterial infection (28), that results in the development of potentially fatal complication known as multiple organ dysfunction syndrome. Hind limb ischemia/reperfusion has been extensively used in our laboratory as a model of systemic inflammatory response syndrome, which closely resembles the acute traumatic and ischemic insult seen in systemic inflammatory response syndrome patients (29). It has been demonstrated that hind limb ischemia/reperfusion causes cellular injury in remote organs (kidney, lung, and myocardium) and contributes to the development of multiple organ dysfunction syndrome (5, 29). As far as we know, there are only a few reports demonstrating testicular remote injury following muscle ischemia/reperfusion injury (8). The results of Takhtfooladi et al. (8) indicated that skeletal muscle ischemia/reperfusion induces severe testicular damage and N-acetylcysteine has protective effects on testicular injury after hind limb ischemia/reperfusion. Their data supported this view that temporary occlusion of the femoral artery induced testicular injury in rats (8). Previous studies indicate that inflammatory response and injury to remote organs can be caused by the systemic release of the pro-inflammatory mediators and free oxygen radicals upon reperfusion of ischemic limbs (5, 30). Despite decades of research

Table 1 - The tissue CAT and MPO activities and levels of SOD and MDA in testicular tissues and score of testicular histological changes and muscle wet/dried weight (W/D) ratio.

	Ischemia/Reperfusion	Ischemia/Reperfusion + Pentoxifylline
SOD (U/mg protein)	4.016±0.28	8.12±0.13*
CAT (k/g protein)	81.03±4.17	98.10±2.73*
MPO (U/g tissue)	1.03±0.20	0.61±0.08*
MDA (nmoL/g tissue)	2.74±0.33	1.85±0.21*
Histological Grading	2.30±0.67	1.40±0.51*
W/D Ratio	2.20±0.13	1.64±0.09*

*P < 0.05 compared with the ischemia-reperfusion group.

Figure 1 - Light microscopic view of testis tissues from IR group showing coagulative necrosis with loss of seminiferous tubule epithelium, edema and sloughed germinal cells. H&E staining; magnification of 10 × 10.

Figure 2 - Light microscopic view of testis tissues from PTX treated group showing normal seminiferous tubule morphology with orderly arrangement of germinal cells. H&E staining; magnification of 10 × 10.
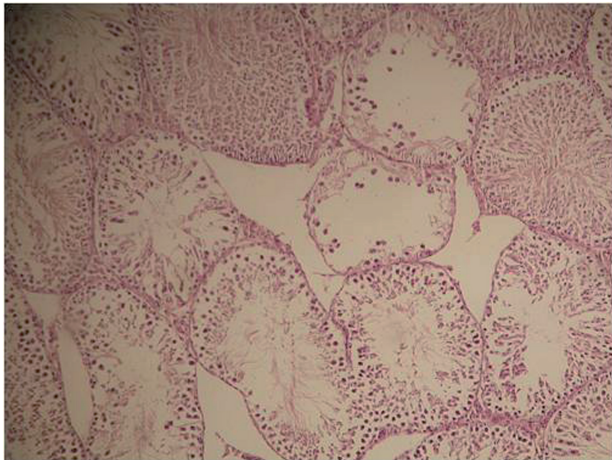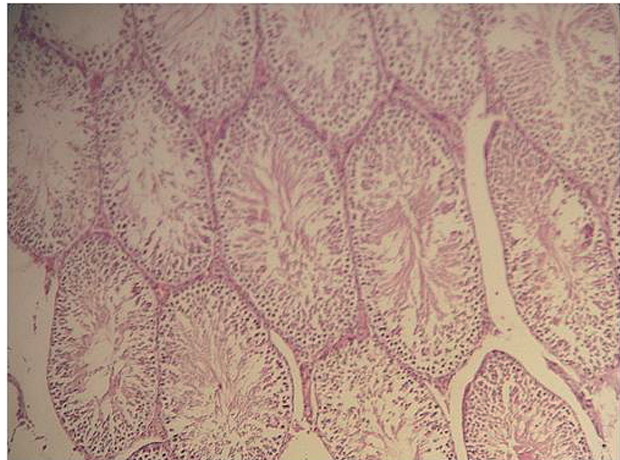

in this area, ischemia/reperfusion injury remains a clinically challenging problem.

PTX is one of the phosphodiesterase inhibitors that have been reported to increase intracellular cyclic AMP and reduce superoxide anion production by both monocytes and polymorphonuclear cells dose dependently in vitro (31). Endres et al. (32) indicated that PTX led to a marked increase in cyclic AMP levels, whereas cyclic GMP levels were only marginally elevated in lipopolysaccharide stimulated human monocytes. PTX has received considerable attention with respect to its action on leukocytes in many organs (33-35). Reignier et al. (19) reported that after reperfusion, myeloperoxidase activity and blood neutrophil count were lower with PTX than with saline, and changes in the filtration coefficient were correlated to the percent changes in blood neutrophils during reperfusion. They suggested that this effect may be mainly caused by a decrease in sequestration of neutrophils in the lung during reperfusion. Their group also reported that PTX prevented endothelial injury during ischemia/reperfusion by decreasing neutrophil sequestration in isolated perfused rat and rabbit lungs and in pigs after left lung allotransplantation (33).

Previous studies have also confirmed the potential antioxidant effects of PTX (36-38). Recently administered PTX was shown to protect against ischemia/reperfusion injuries in local and remote organs and it was suggested that this protective effect may be due to its ability to inhibit of free radical generation (12, 20). There is growing evidence regarding its beneficial effects in ameliorating testicular ischemia/reperfusion injury (39). In an experimental study Savas et al. (39) suggested that the administration of PTX at a dose of 50 mg/kg 15 min. before spermatic cord torsion may have a protective effect in rat experimental testicular torsion/detorsion models. Their results suggest that pentoxifylline treatment attenuates reperfusion damage on both sides, possibly with its effects on blood flow and neutrophils (39). This observation was supported by Pozor et al. (40), who demonstrated that PTX may be a potential protective agent for preventing the negative changes related to oxidative stress in testicular injury caused by spermatic cord torsion in miniature horse stallions. However, the protective effect of PTX on testes from skeletal muscle ischemia/reperfusion injury has not been studied to date. In the current study, we tested the hypothesis that PTX could protect the testes from remote organ injury after skeletal muscle ischemia/reperfusion.

Oxidative stress is associated with an increased rate of cellular damage induced by oxygen and oxygen-derived oxidants, commonly known as reactive oxygen species (41, 42). The major targets of reactive oxygen species are membrane lipids, in a process known as lipid peroxidation.

It is also acknowledged that testicular tissues and spermatozoa are very sensitive to reactive oxygen species attack and lipid peroxidation. The susceptibility of testicular tissues to oxidation is attributed to the high polyunsaturated fatty acid content of sperm membranes (42, 43). Many tissues contain powerful endogenous scavengers that provide protection against free radical damage, including SOD, CAT, glutathione peroxidase, ascorbic acid and α-tocopherol (44). Sikka et al. (45) have reported that adequate levels of antioxidants such as SOD, CAT and possibly glutathione peroxidase and reductase, maintain the scavenging potential in gonads and seminal fluids, which is referred to as oxidative stress status. Tissue CAT and MPO activities, and SOD and MDA levels are considered to indicate oxidative stress. In the present study, SOD level and CAT activity were decreased significantly in IR group compared with IR+PTX group. The tissue MDA level and MPO activity in testes were increased significantly in IR group compared with IR+PTX group. According to the observations of this study, the histopathological injury score was significantly decreased in IR+PTX group compared with that of IR group. In the PTX treated group, histopathological features such as edema, congestion, hemorrhage, and necrosis of the germinal cells were markedly less than in IR group. The findings in this study indicate that reperfusion of the ischemic limb leads, within 24h of reperfusion, to a systemic response as demonstrated by the biochemical and histological impairment of the testis. Also, data on CAT and MPO activity, and SOD and MDA levels suggested a protective effect of PTX against testicular remote injuries after unilateral hind limb ischemia/reperfusion. These data together with previous findings (39) confirm the potent anti-oxidation capacity of PTX. These data also support the concept that PTX may be an effective therapeutic adjunct to reperfusion injury. The clinical relevance of this manuscript refers to the previous use of PTX in situations requiring procedures ischemia with reperfusion to reduce or prevent distant organs damage. Therefore, the long-term effect of PTX warrants further investigation.

CONCLUSIONS

The results of this study showed that unilateral hind limb ischemia/reperfusion induced testicular injury in rats. Nevertheless, PTX administration significantly decreased testes injury induced by skeletal muscle ischemia/reperfusion according to our histological and biochemical findings. PTX is already in clinical use for the treatment of vascular diseases and these results suggest the possibility of clinical application of PTX in testicular remote organ injury following skeletal muscle ischemia/reperfusion. Further studies are needed to clarify clinical usefulness of this agent.

CONFLICT OF INTEREST

None declared.

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