

The protective role of curcumin on testicular tissue after hindlimb ischemia reperfusion in rats

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Abstract

Purpose The aim of this study was to investigate the role of curcumin in remote testicular injury caused by hindlimb ischemia reperfusion (IR).

Materials and methods Forty male Wistar rats were allocated to four groups: sham (G1), sham + curcumin (G2), IR (G3) and IR + curcumin (G4). Curcumin 200 mg/kg was administered intraperitoneally 2 h prior to IR induction. Lower extremities were subjected to IR induced by infrarenal aortic occlusion for 2 h, followed by 6 h of reperfusion. The rats were euthanized and the testes were removed. Glutathione peroxidase (GPx), superoxide dismutase (SOD), malondialdehyde (MDA), catalase (CAT) and myeloperoxidase (MPO) activities and histopathological damage scores were determined in right testicular tissues. Left testes were used for wet/dry weight ratio measurement.

Results Activities of SOD and CAT in testicular tissues were significantly decreased by IR, but curcumin pretreatment increased these levels ($P < 0.05$). MPO activity in testicular tissues in the G3 was significantly higher than in the G4 ($P < 0.05$). Significantly increased MDA levels in testicular tissues by IR were decreased by curcumin pretreatment ($P < 0.05$). Testis tissues showed a significant increase in GPx activity compared to the IR group when curcumin was applied. The wet/dry weight ratio of testicular tissues

in the G3 was significantly higher than in the other groups ($P < 0.05$). In addition, specimens from the G3 had a significantly greater histological injury than those from the G4 ($P < 0.05$). There were no significant differences in tissue MDA, MPO, SOD, CAT and GPx activities, histological changes and wet/dry weight ratio between the G1, G2 and G4.

Conclusions According to the findings, we conclude that curcumin has preventive effects in the testicular injury induced by hindlimb IR in rats.

Keywords Curcumin · Testis · Remote organ · Ischemia reperfusion · Oxidative stress

Introduction

Ischemia reperfusion (IR) injury is a complex phenomenon often seen in clinical events. This injury is not only limited to organs directly affected by IR but also found in distant organs. It is well recognized that an IR injury is characterized by an increase in reactive oxygen species (ROS) [1]. ROS stimulate the release and the formation of various inflammatory mediators with powerful chemotactic potential. These mediators lead to leukocyte activation and endothelial adhesion molecule expression and vascular endothelial damage in remote organs [1]. At the same time, ROS are capable of reacting with proteins, nucleic acids and lipids leading to lipid peroxidation of biological membranes. Skeletal muscle IR is associated with a systemic inflammatory response and has effects on remote organs such as liver [2], lung [3], kidney [4] and testis [5] and their structure and function.

Many chemicals have been tested to attenuate IR injury in target and remote organs. Among them, curcumin is an

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orange-yellow component of turmeric (*Curcuma longa*), which is derived from the rhizome of the plant [6]. It has many biological and pharmacological activities, including anti-inflammatory, antimicrobial, antiviral, antifungal, antioxidant, chemosensitizing, radiosensitizing and wound healing activities [7]. It has been also demonstrated that curcumin has protective role in many experimental IR injury models such as cardiac [8], kidney [9] and hepatic [10]. There is growing evidence regarding its beneficial effects in ameliorating testicular IR injury [11]. However, the effects of curcumin on remote testicular injury caused by skeletal muscle IR are not clear. The aim of this study was to investigate the effects of curcumin on the testis as a remote organ after performing hindlimb IR, by assessing histological and biochemical parameters of the testis tissue.

Materials and methods

Animals

All experimental procedures were performed according to the guidelines for the ethical treatment of experimental animals and approved by the Islamic Azad University College of Veterinary Sciences, Animal Care and Use Local Ethics Committee.

The experiments were performed on 40 healthy mature male Wistar rats ranging in weight from 230 to 250 g. The animals were kept at a temperature of 22 ± 2 °C with a 12-h dark/light cycle. Standard commercial rodent chow pellets and filtered tap water were allowed ad libitum.

Experimental groups

Rats were randomly allocated into four experimental groups as follows:

1. Sham (G1) (n 10): Animals were exposed to midline laparotomy without clamping the infrarenal aortic.
2. Sham plus curcumin (G2) (n 10): Curcumin was given intraperitoneally (i.p.) 2 h prior to surgery. Thereafter, animals were exposed to midline laparotomy without clamping the infrarenal aortic.
3. IR (G3) (n 10): Rats were exposed to midline laparotomy with occlusion of the infrarenal aortic for 2 h, followed by 6 h of reperfusion period.
4. Curcumin plus IR (G4) (n 10): Curcumin was administered i.p. 2 h prior to IR induction. Thereafter, animals were exposed to midline laparotomy with occlusion of the infrarenal aortic for 2 h, followed by 6 h of reperfusion period.

Experimental protocol

Curcumin 200 mg/kg (Sigma-Aldrich Co., St. Louis, MO, USA) dissolved in dimethyl sulphoxide (DMSO) was administered i.p. 2 h prior to IR induction. During the surgical procedures, anesthesia was induced and then maintained with ketamine hydrochloride 10 % and xylazine hydrochloride 2 % (50, 3 mg/kg, i.m., respectively), as needed. During the surgical procedures, the body temperature was maintained with a heating pad. Then, animals were given heparin of 1000 U/kg via the jugular vein. After the surgical preparation using aseptic techniques, a midline laparotomy was performed and then the abdominal aorta was exposed through the incision. After the exploration of the abdominal aorta, a microvascular clamp was placed on the infrarenal abdominal aorta for 2 h. At the end of trial, the clamps were removed to allow reperfusion. The abdominal wound was routinely closed with 3/0 nylon sutures. After 6 h, the rats were euthanized by overdose of pentobarbital injection (300 mg/kg, i.p.), and testicular tissue samples were harvested for histological and biochemical examinations.

Preparation of testicular tissue homogenates

The sample of right testicular tissues was 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 2 min at $11,200 \times g$. The homogenate was centrifuged at $3500 \times g$ (4 °C) for 60 min, and supernatant was obtained. The levels of myeloperoxidase (MPO) were determined in the supernatant, and malondialdehyde (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 $3500 \times g$ for 20 min, the clear upper layer was taken and used for superoxide dismutase (SOD) activity determination [12].

SOD activity

The principle of the SOD activity determination method was based on the inhibition of nitroblue tetrazolium reduction described by Sun et al. [13] and modified by Durak et al. [14]. 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 U/mg tissue.

MDA levels

The MDA levels in testicular tissues were analyzed by a method based on the reaction with thiobarbituric acid at 95 °C [15]. 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 532 nm. The results were expressed as nmol/g tissue.

Catalase (CAT) activity

CAT activity was determined according to Aebi's method [16]. The principle of the assay is based on the determination of rate constant k (s^{-1}) of H_2O_2 decomposition at 240 nm. Results were expressed as k (rate constant) per gram of protein (k/g tissue).

MPO activity

Testicular injury was quantified by measuring testicular MPO activity, the activity of infiltrated polymorphonuclear leukocytes, using a protocol modified from the previous report [17]. MPO activity was determined after adding O-dianisidine dihydrochloride and hydrogen peroxide. The MPO activity was expressed as U/g tissue.

Glutathione peroxidase (GPx) activity

Tissue GPx activity was measured using the modified method of Paglia and Valentine [18]. Using glutathione as a reducing reagent, the GPx enzymes catalyze the reduction of hydrogen peroxide to water. In the assay, oxidized glutathione is reduced to glutathione by the enzyme glutathione reductase, which oxidizes nicotinamide adenine dinucleotide phosphatase, reduced form (NADPH) to NADP in the catalytic cycle. The change in absorbance at 340 nm resulting from the oxidation of NADPH is the basis for quantitating tissue GPx activity. Tissue GPx activity was expressed as U/g tissue.

Histological evaluation

All testis samples were fixed in Bouin's solution for 24–48 h and embedded in paraffin. Tissue sections were stained with hematoxylin/eosin (HE) and observed under a light microscope. An experienced pathologist who was blinded to the experiment and data examined the samples histopathologically. A 4-level grading scale similar to that of Cosentino et al. [19] was used to quantify histological injury. Grade 1 showed normal testicular architecture with an orderly arrangement of germinal cells. Grade 2 injury showed less ordered, noncohesive germinal cells and closely packed seminiferous tubules. Grade 3 injury exhibited disordered, sloughed germinal cells with shrunken, pyknotic nuclei and less distinct seminiferous tubule borders. Grade 4 injury defined seminiferous tubules that were closely packed with coagulative necrosis of the germinal cells.

Wet/dry weight assay

The wet/dry weight ratio, the tissue edema index, was measured to evaluate testicular injury. Briefly, freshly left harvested testes were weighed, placed in an oven for 24 h at 60 °C and weighed again after drying [17]. The wet/dry weight ratio was then calculated.

Statistical analysis

All statistical analyses were performed using SPSS for Windows, 16.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as the mean \pm standard deviation (SD). Analysis of variance was used for statistical analysis of the data

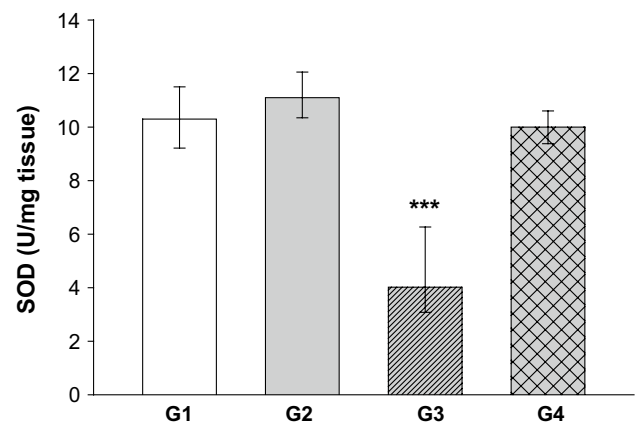


Fig. 1 Superoxide dismutase (SOD) activities in testicular tissues from rats subjected to a different procedure. Sham (G1), sham + curcumin (G2), IR (G3) and IR + curcumin (G4). Data expressed as mean \pm SD. *** $P < 0.001$ compared with other groups

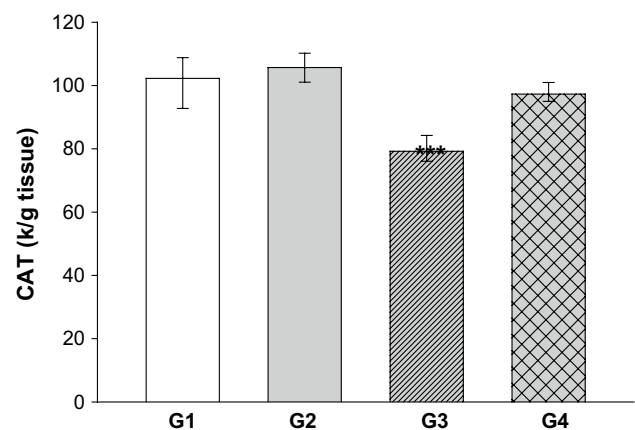


Fig. 2 Catalase (CAT) activities in testicular tissues from rats subjected to a different procedure. Sham (G1), sham + curcumin (G2), IR (G3) and IR + curcumin (G4). Results were expressed as k (rate constant) per gram of protein (k/g tissue). *** $P < 0.001$ compared with other groups

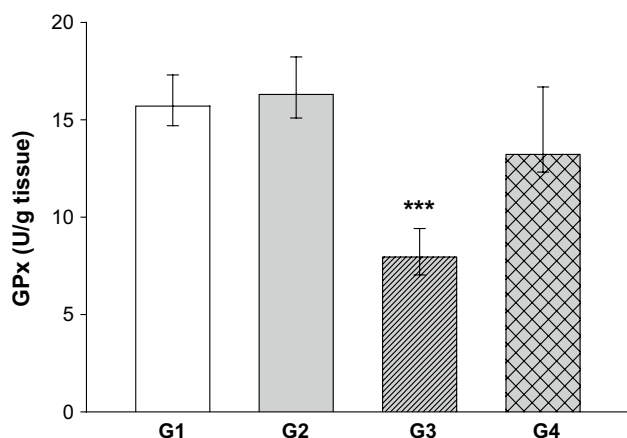


Fig. 3 Glutathione peroxidase (GPx) activities in testicular tissues from rats subjected to a different procedure. Sham (G1), sham + curcumin (G2), IR (G3) and IR + curcumin (G4). Data expressed as mean \pm SD. *** $P < 0.001$ compared with other groups

among all groups. Multiple comparisons were made using Tukey's procedure, with $P < 0.05$ considered statistically significant.

Results

All rats survived without major complications. Significant decreases were found in the SOD, CAT and GPx activities in the G3 compared with other groups ($P < 0.05$) (Figs. 1, 2, 3 respectively). The tissue levels of MDA increased in the G3 in comparison to the other groups ($P < 0.05$) (Fig. 4). MPO activity in testicular tissues in the G3 was significantly higher than in the other groups ($P < 0.05$) (Fig. 5). The wet/dry weight ratio of testicular tissues in the G3 was significantly higher than in the other groups ($P < 0.05$) (Fig. 6). In addition, specimens from the G3 had a significantly greater histological injury than the G4 ($P < 0.05$) (Fig. 7). There were no significant differences in tissue MDA, MPO, SOD, CAT and GPx activities, histological changes and wet/dry weight ratio between the G1, G2 and G4 ($P > 0.05$).

Discussion

Ischemic injury frequently occurs in the hindlimb because of the hypoperfusion period related to aortic clamping during the abdominal aortic surgery. During ischemia, muscle cells cannot keep their membrane integrity and this causes releasing of calcium, phospholipid A2, formation of polyunsaturated fatty acids and fatty acid radicals. If the oxygenation is re-established at that stage of ischemia, fatty acid radicals react with oxygen and undergo lipid

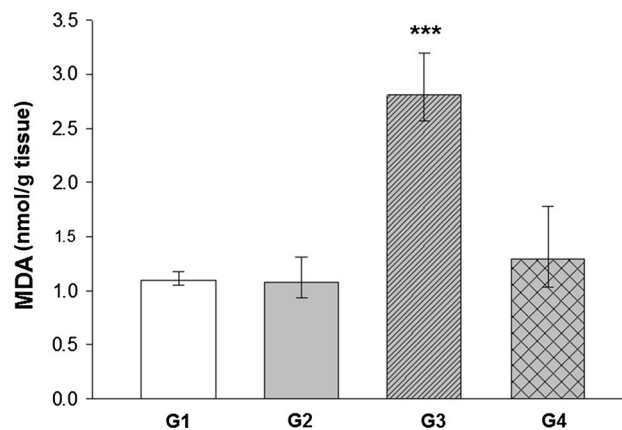


Fig. 4 Mean values of tissue malondialdehyde levels of all groups. Sham (G1), sham + curcumin (G2), IR (G3) and IR + curcumin (G4). Data expressed as mean \pm SD. *** $P < 0.001$ compared with other groups

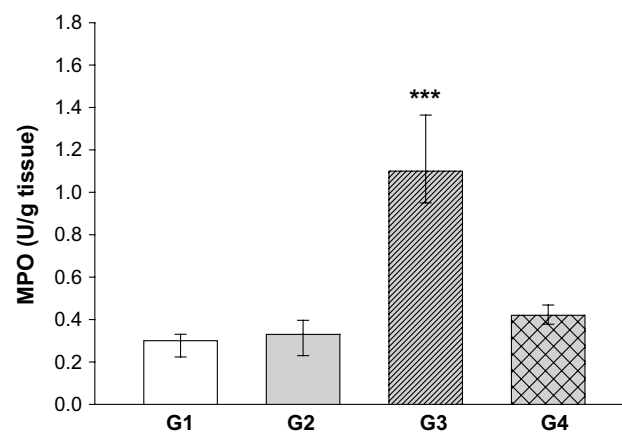


Fig. 5 Myeloperoxidase (MPO) activities in testicular tissues from rats subjected to a different procedure. Sham (G1), sham + curcumin (G2), IR (G3) and IR + curcumin (G4). *** $P < 0.0001$ compared with other group

peroxidation reaction. This reaction increases the membrane permeability and also stimulates chemotaxis of leukocytes, which release oxygen-derived free radicals and proteolytic enzymes when activated. Activated leukocytes release a variety of inflammatory mediators, including cytokines, neutrophil proteases and ROS. All of these products cause damage to adjacent endothelial cells, and they have been thought to play key roles in tissue injury [20]. Reperfusion injury develops on remote organs and leads to tissue injuries such as lungs, heart, liver, kidneys, heart, testes, and even death resulting from systemic toxic effects of reperfusion products [21, 22].

The role of oxidative damage in the testes following hindlimb IR injury is well established [5]. In this study, we also showed that testis, as a remote organ, is affected by IR

of the lower extremities. As far as we know, despite many studies concerning the prevention of IR injury, this is the first study in which curcumin was used for preventing IR injury in testis remote organ.

Curcumin is an antioxidant derived from turmeric (*C. longa*, *Zingiberaceae*) and has been known since ancient times to possess therapeutic properties. It has been reported to scavenge oxygen free radicals and to inhibit lipid peroxidation and protect the cellular macromolecules, including DNA from oxidative damage [23, 24]. Accordingly, curcumin has been shown to efficiently prevent lipid peroxidation in rat hepatocytes during oxidative stress [25]. Other studies have confirmed its ability to scavenge oxygen free radicals to increase intracellular glutathione concentrations and to prevent lipid peroxidation [26, 27]. Normal cells are supposed not to be affected by curcumin [28]. A recent investigation demonstrated a protective effect of curcumin treatment during ischemia of testes [11]. Our study confirmed that treatment with

curcumin might attenuate testicular injury secondary to temporary clamping of the hindlimb. According to these results, MDA levels in testicular tissues were found to be higher in the IR group. MDA is, in particular, released as a result of toxic effects of active oxygen radicals which destroy unsaturated fatty acids in the cell membrane. Neutrophil infiltration might be regarded as another source of free radicals in the ischemic tissue since activation of neutrophils results in the production and release of potentially toxic metabolites [1, 29]. In this study, tissue MDA levels were clearly decreased by curcumin, but its mechanism is not clear. Curcumin may eliminate free oxygen radicals or may increase antioxidant enzyme activity and prevent the inhibition of these enzymes. Since antioxidant properties of curcumin are well documented, the decrease in MDA levels in the curcumin with IR group is probably due to its antioxidant effect. A decrease in MDA with curcumin treatment in the IR condition has been shown in previous studies [6, 30]. Leukocytes exposed to ischemic tissue may re-enter the systemic circulation in an activated state upon reperfusion. These activated neutrophils have also been implicated as mediators of IR-induced distant organ damage [31]. Higher MPO activity was detected in testes in the IR group. Increase in MPO activity in testicular tissue may reflect activation of neutrophils in the IR condition. In this study, treatment with curcumin reduced MPO activity in testes. The histological injury score was significantly decreased in the G4 compared with that of the G3. In the G4, histopathological features such as edema, congestion, hemorrhage, and necrosis of the germinal cells were markedly less than in G3.

According to the histological and biochemical findings, we demonstrated that administration of curcumin protects the testes from IR injury. Until now, studies with curcumin effect have mainly concentrated on local IR injury. As suggested by our data, curcumin is also effective in protecting remote organ injury caused by increased systemic ROS and neutrophil accumulation which results from the IR injury.

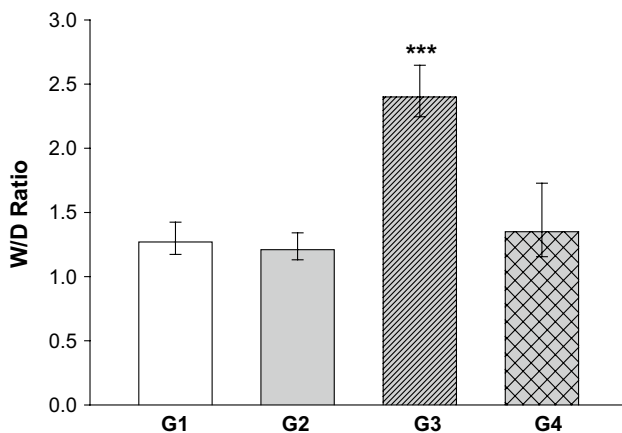


Fig. 6 Wet/dry weight ratio, the tissue edema index, was measured to evaluate testicular injury in all groups. Sham (G1), sham + curcumin (G2), IR (G3) and IR + curcumin (G4). Data expressed as mean ± SD. *** $P < 0.001$ compared with other groups

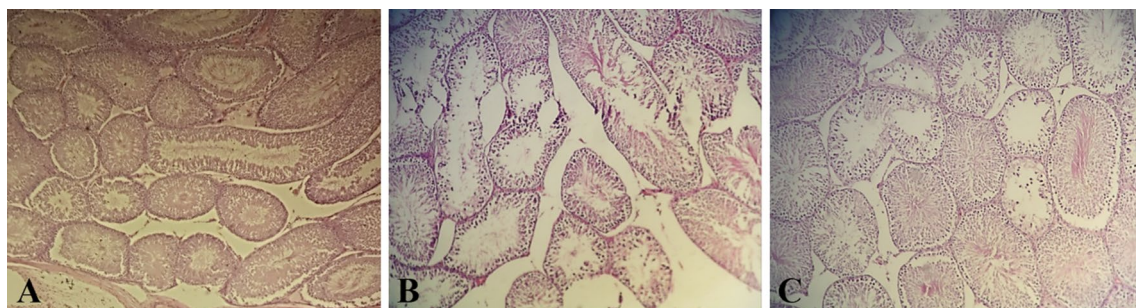


Fig. 7 Light microscopy of testicular tissue in different groups. H&E: **a** in sham, normal testicular architecture was seen; **b** after ischemia reperfusion, severe testicular damage was noted; **c** curcumin treatment prevented testicular damage

Conclusions

In conclusion, it is important to prevent IR injury, and curcumin would be a useful therapeutic agent for the treatment of a variety of conditions associated with tissue ischemia.

Compliance with ethical standards

Conflict of interest MA. Takhtfooladi declares that he has no conflict of interest. A. Asghari declares that he has no conflict of interest. HA. Takhtfooladi declares that he has no conflict of interest. S. Shabani declares that he has no conflict of interest. H. Hajizadeh declares that he has no conflict of interest.

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

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