

## Protective Effects of Leptin on Ischemia/Reperfusion Injury in Rat Bladder

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### Abstract

The aim of the study was to evaluate protective effects of exogenous leptin on ischemia/reperfusion (I/R)-induced injuries to the urinary bladder tissue and to investigate the effect on tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) levels and apoptotic cells during I/R injury. Bladder I/R injury was induced by abdominal aorta occlusion by ischemia for 45 min, followed by 60 min of reperfusion in rats. The rats were divided into three groups: control (n = 8 + 8), I/R (n = 8 + 8) and I/R+leptin group (n = 8 + 8). The rats in the I/R+leptin group were treated intraperitoneally with leptin (10  $\mu$ g/kg) 60 min prior to ischemia induction. At the end of the reperfusion period, urinary bladders of the first eight rats from each group were removed for TUNEL staining processing while the others were removed for biochemical analyses for MDA and TNF- $\alpha$  levels. In the I/R group, the ratios of TUNEL-positive nuclei were higher than the control and the I/R+leptin groups. The MDA and TNF- $\alpha$  levels of the bladder tissue in the I/R group were higher than the control and leptin-treated groups. TUNEL-staining and biochemical studies revealed that leptin has a protective effect on urinary bladder I/R injury.

**Key Words:** ischemia/reperfusion injury, oxidative stress, leptin, rat, urinary bladder, apoptosis

### Introduction

Ischemia due to arterial occlusion, shock or organ transplantation is a common cause of cell death or organ failure. Reperfusion, although essential for tissue survival, aggravates tissue damage caused by ischemia (9, 15). Reperfusion on ischemic tissue generates reactive oxygen species causing lipid peroxidation of cellular membranes, and alters various cellular functions (13). Although several mechanisms have been proposed to explain the pathogenesis of ischemia/reperfusion (I/R) injury, attention has been focused on the role of reactive oxygen species (ROS) such as superoxide radical ( $O_2^{\cdot-}$ ), hydroxyl radical (OH), hydrogen peroxide ( $H_2O_2$ ) and inflammatory

leukocytes (12). During ischemia, interruption of blood supply and the lack of oxygen lead to anaerobic metabolism with a loss of energy substrates and the accumulation of hypoxanthine within the ischemic cells. Reperfusion stimulates the transformation of hypoxanthine and xanthine to uric acid and excessive production of ROS. ROS can induce cell injury through lipid peroxidation in mitochondrial, lysosomal and plasma membranes by altering membrane structure and functions (12, 21).

The major cause of urinary bladder failure is I/R injury which may also be involved in the development and progression of some forms of urinary bladder disease. It is known that normal bladder function depends on the integrity of its autonomic innervation,

cellular structure and metabolism (26), and is maintained by an adequate supply of oxygen and nutrients *via* the circulation system. Studies have shown that partial obstruction of bladder outlet in different species induces a relative decrease in bladder blood flow (16). *In vivo* and *in vitro* studies shown that ischemia-induced bladder functional changes cause significant bladder dysfunction and trigger decreased contractile responses to all forms of stimulation (1, 3, 26). When ischemia is temporary or reversible, it is followed by reperfusion. It is reported that reperfusion results in a more severe injury in the rat bladder than does ischemia alone. It is known that reperfusion contributes to cell damages induced by partial outlet obstruction and reversible ischemia *via* free-radical formation (2, 14).

On the other hand, oxidants released during reperfusion stimulate transcription factors which are related to tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) expression (6). An increased level of TNF- $\alpha$  has been correlated with disease severity (5). Ischemia stimulates the release of TNF- $\alpha$  from both local and remote organ sites and it is an important mediator of neutrophil-dependent tissue damage (17, 22). TNF- $\alpha$  stimulates the release of other inflammatory mediators including interleukin-1, platelet-activating factor, oxygen radicals, nitric oxide, and prostaglandins (17).

Although several mechanisms have been proposed to explain the pathogenesis of I/R injury, and ischemic urinary bladder failure is well documented, attention has been focused on the role of post-ischemic inflammatory cytokines, oxidative stress, and apoptosis (programmed cell death). However, an increasing body of evidence now indicates that apoptosis plays an essential role in cell death after I/R (24). Apoptosis, induced by stresses and insults, is important in maintaining homeostasis and proper function in many systems. But no information is available yet about the reversibility of urinary bladder dysfunction and apoptosis induced by I/R.

Leptin, a circulating hormone secreted by adipocytes, influences body weight homeostasis through effects on food intake and energy expenditure and plays an important role in the regulation of fatty acid metabolism in liver, pancreas, skeletal muscle and in the heart (8, 23). Additionally, there are many studies indicating that leptin treatment significantly decreases ischemic intestinal injury and has a potent gastro-protective activity against I/R-induced gastric lesions (4, 10). Moreover, we had shown in our recent study that leptin had a protective effect on renal and gastric tissues and on cultured cardiac myocytes (7-9). In the literature, we did not find any study indicating the effects of leptin on urinary bladder tissue. Therefore, we planned the present study with the aim of evaluating

the protective effect of exogenous leptin on I/R-induced urinary bladder tissues and to investigate TNF- $\alpha$  levels and apoptotic cells during I/R injury.

## Materials and Methods

### Animals

Sprague Dawley rats (n = 48) weighing 200-250 g were supplied by the Eskişehir Osmangazi University Experimental Research Center, Eskişehir, Turkey. Rats were housed in polycarbonate cages at a temperature ( $21 \pm 1^\circ\text{C}$ ) and humidity (45-55%) controlled room that was maintained on a 12/12 reversed light cycle. The rats were fed with a standard rat chow (Oguzlar Yem, Eskişehir, Turkey) and allowed to drink water *ad libitum*. This study was approved by The Eskişehir Osmangazi University Institutional Local Animal Care and Use Committee (date 08.02.2007 No: 12).

### Experimental Groups

Animals were divided into three groups, control (n = 8 + 8), I/R (n = 8 + 8) and I/R+leptin group (n = 8 + 8). The first eight rats from each group were selected for histological examination and the other eight rats were used for biochemical determinations. Leptin (10  $\mu\text{g}/\text{kg}$ ) was administered intraperitoneally (i.p.) 30 min prior to ischemia process. The rats were anesthetized with thiopental (50 mg/kg, i.p.) and were placed on a heating pad to maintain constant body temperature. Under aseptic conditions, a midline incision was made, and a non-traumatic vascular clamp was applied to the abdominal aorta for 45 min and then allowed for reperfusion for 60 min. At the end of the reperfusion period, the urinary bladders of the first eight rats from each group were removed for TUNEL staining while the others were removed and kept frozen at  $-80^\circ\text{C}$  until biochemical analyses for MDA and TNF- $\alpha$  levels.

### In Situ Apoptosis Detection

Firstly, urinary bladders were carefully excised and fixed in neutral buffered formalin for histologic analyses. After the fixation, the tissues were embedded in paraffin. Serial sections (5  $\mu\text{m}$ ) were prepared for each of the paraffin blocks. On the average, 50 sections were collected per rat. Sections were stained with hematoxylin and eosin and for apoptosis with TUNEL staining. Digital images were obtained by an Olympus BX-61 (Olympus America Inc. NY, USA) microscope with a DP70 digital camera.

Cleavage of genomic DNA during apoptosis may yield double-stranded, low molecular-weight

DNA strand breaks that can be labeled by terminal deoxynucleotidyl transferase (TdT) which catalyzes polymerization of labeled nucleotides to free 3'-OH ends in an template-independent manner (TUNEL reaction). The quantity of apoptotic cell death was evaluated using an *in situ* cell death detection kit (Cat. no.S7101; Chemicon International-ApopTag Plus Peroxidase Kits, USA). Firstly, the serial sections were deparaffinized three times in xylene. The sections were rehydrated through a series of decreasing concentrations of ethanol before the slides were washed in PBS (phosphate-buffered saline). The sections were subjected to partial digestion with proteinase K (20 µg/ml) (Merck) at room temperature for 15 min and were washed 2 times in PBS. Thereafter, the sections were incubated at room temperature in an equilibration buffer. The tissue sections were incubated at 37°C for 60 min with the TUNEL reaction mixture (70% reaction buffer, 30% TdT enzyme) in a humidified chamber in the dark. The slides were agitated and incubated in the stop/wash buffer for 10 min at room temperature. After the incubation, the slides were washed 3 times with PBS (phosphate-buffered saline). Then, anti-digoxigenin conjugate was applied to the slides and incubated in a humidified chamber for 30 min at room temperature. The slides were washed 4 times with PBS. DAB (diaminobenzidine) was added onto the slides for 10 min, and the slides were washed in distilled water three times before drying. For analysis of apoptotic cells, two independent observers evaluated 25 optical fields for each 10 sections per animal chosen randomly using a ocular micrometer by light microscopy.

#### Biochemical Analyses

Urinary bladder tissues were homogenized in 0.1 M sodium phosphate buffer by using a homogenizer (Ultra Turrax IKA T18 basic, Wilmington, NC, USA). Homogenized sample were centrifuged at 10,000 rpm for 10 min, and the supernatant fractions were analyzed by means of MDA and TNF- $\alpha$  levels.

The lipid peroxidation level was measured by determining the end product of lipid peroxidation, MDA, by the thiobarbituric acid method (19). TNF- $\alpha$  was measured by using an enzyme-linked immunosorbent assay (ELISA) kits supplied from R&D Systems (Minneapolis, MN, USA).

#### Statistical Analyses

Results are expressed as means  $\pm$  SD. SPSS version for Windows 13.0 was used to evaluate the data. Statistical analysis was performed by using One way ANOVA and Tukey HSD multiple comparison test. Rejection of the null hypothesis was set at  $P < 0.05$ .

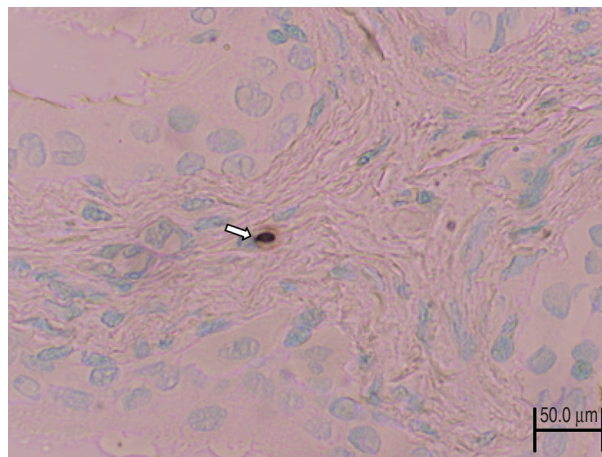


Fig. 1. Few apoptotic cells in normal urinary bladder. TUNEL-positive cell, indicated by an arrow, was rarely seen in control group in lamina propria of urinary bladder. (Original magnification  $\times 40$ )

## Results

#### *In Situ Apoptosis Detection*

The results of the TUNEL staining performed to detect apoptotic cells in the rat urinary bladders are shown in figures and table. In the control group, each of the bladder layers (urothelium, lamina propria and detrusor) showed few apoptotic cells suggesting that this process occurred at a very limited rate in normal urinary bladder (Fig. 1).

In the I/R group, the ratios of TUNEL-positive nuclei were higher than the control group for each of the layers (Table 1). In this group, TUNEL-positive nuclei were primarily detected in the tunica mucosa (Fig. 2), submucosa and muscularis. On the other hand, for all of the layers, the ratios were decreasing in leptin-treated group (Figs. 3 and 4).

#### *Biochemical Determination*

The MDA levels of the bladder tissue in the I/R group ( $7.9 \pm 1.1$  nmol/mg protein) were higher than those of the control group ( $4.2 \pm 0.5$  nmol/mg protein;  $P < 0.05$ ). Leptin treatment of the I/R group decreased the MDA levels compared to I/R alone ( $4.0 \pm 0.3$  nmol/mg protein;  $P < 0.05$ ) (Fig. 5).

On the other hand in the I/R group, the TNF- $\alpha$  levels of the bladder tissues were increased when compared to the control group ( $23.6 \pm 4.5$  and  $5.5 \pm 0.6$  ng/g protein respectively;  $P < 0.001$ ). Leptin treatment of the I/R group caused a marked decrease in the TNF- $\alpha$  values compared to I/R alone ( $8.6 \pm 1.6$  ng/g protein;  $P < 0.01$ ) (Fig. 6).



**Table 1. Ratio of TUNEL-positive cells in bladder layers (in %)**

	Control	I/R	I/R + Leptin
Urothelium	0.08 ± 0.01	0.73 ± 0.10*	0.30 ± 0.04 <sup>+</sup>
Lamina propria	0.18 ± 0.03	1.34 ± 0.15*	0.41 ± 0.05 <sup>+</sup>
Detrusor	0.06 ± 0.01	5.23 ± 0.62*	0.66 ± 0.10 <sup>+</sup>

Data are shown as means ± SEM.

\*Significantly different from control ( $P < 0.001$ ).

<sup>+</sup>Significantly different from I/R ( $P < 0.001$ ).

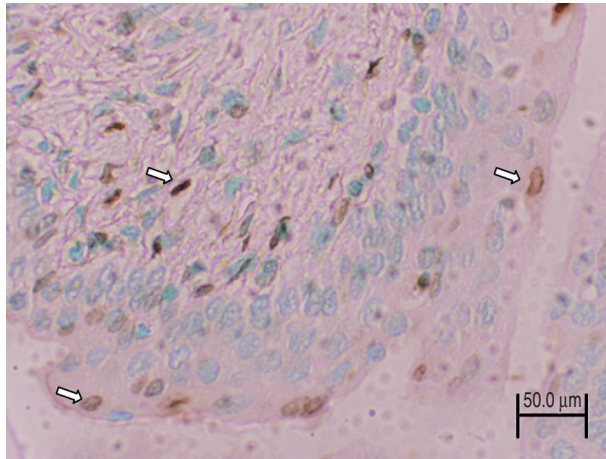


Fig. 2. Effects of ischemia-reperfusion on urinary bladder. In the I/R group, there are many TUNEL-positive cells (arrows) in the lamina propria and epithelium of urinary bladder. (Original magnification ×40)

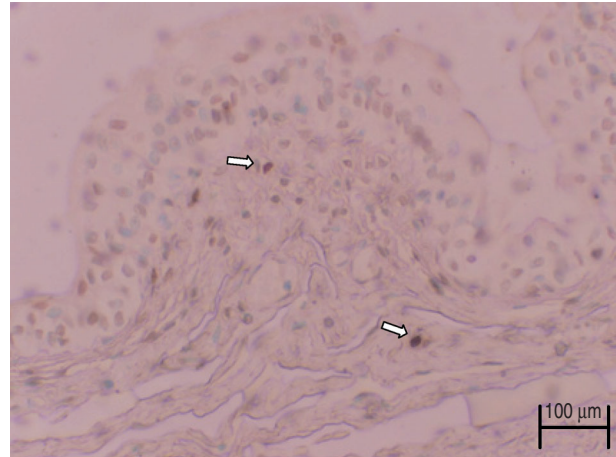


Fig. 4. Effects of leptin treatment in the I/R + Leptin group on urinary bladder. Limited number of TUNEL-positive cells (arrows) in the lamina propria in the I/R+leptin group. (Original magnification ×20)

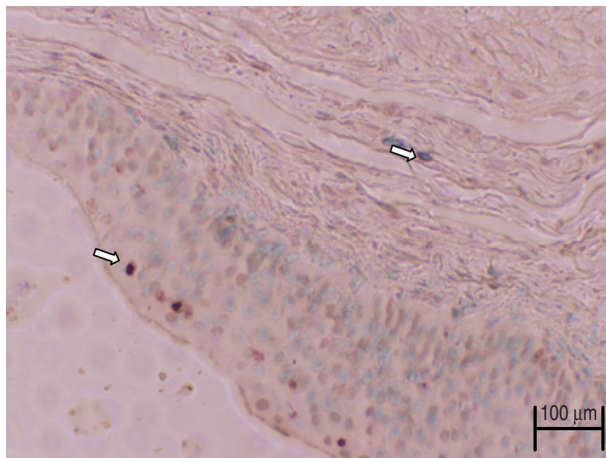


Fig. 3. Effects of leptin treatment in the I/R + Leptin group on urinary bladder. Limited number of TUNEL-positive cells (arrows) in the epithelium and lamina propria in the I/R+leptin group. (Original magnification ×20)

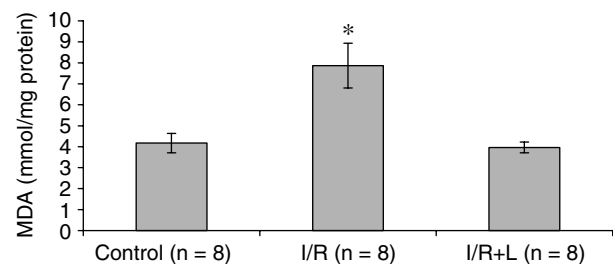


Fig. 5. Effect of leptin on tissue MDA levels. \* $P < 0.05$ , significantly different from the control and leptin treatment groups.

## Discussion

There are no data on the effects of leptin on oxidative damage and apoptotic mechanism caused

by I/R injury in the rat urinary bladder tissue. The present study is the first report to evaluate the effects of leptin, the *ob* gene product, on oxidative damage and formation of apoptosis caused by I/R injury in urinary bladder tissues. Our results indicated that leptin significantly protected urinary bladder tissues against oxidative damage and significantly prevented from apoptotic process. While I/R increased the MDA and TNF- $\alpha$  levels and apoptotic cells in urinary bladder tissues, leptin treatment decreased the levels and the ratio of apoptotic cells. It is also known that

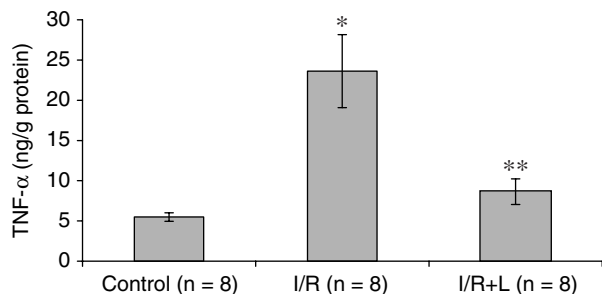


Fig. 6. Effect of leptin on tissue TNF- $\alpha$  levels. \* $P < 0.001$  significantly different from the control group. \*\* $P < 0.01$  significantly different from I/R group.

leptin has protective effects on some tissues such as myocard, renal and gastric tissues due to I/R injury (7-9).

It is well known that, ROS, such as superoxide radical, hydroxyl radical, hydrogen peroxide, play an important role in the formation of I/R injury (26). These radicals cause oxidative damages to biological molecules such as lipids, proteins and DNA and are, therefore, involved in the pathogenesis of I/R-induced urinary bladder damage. We found that I/R of the bladder was associated with a significant generation of ROS compared to the control group. MDA is one of the end products formed due to the decomposition of lipid peroxidation products and is commonly used as a biomarker of oxidative damage (11). In the present study, we observed that MDA levels were increased during I/R compared to the control and leptin treatment groups showing inhibitory effects against I/R-induced oxidative damage in urinary bladder tissues.

TNF- $\alpha$  is an acute-phase reactive protein and a basic medium of immunological regulation, and it is also an inflammatory cytokine with biological effects. Accumulating evidence suggests that local early TNF- $\alpha$  production may play a role in the pathogenesis of I/R injury. Donnahoo *et al.* have reported that TNF- $\alpha$  plays an important role in the pathogenesis of renal I/R injury (6). Similarly, Erkasap *et al.* showed that leptin had a protective effect on I/R-induced renal damage by decreasing TNF- $\alpha$  levels (9). In our study, leptin treatment decreased I/R-induced TNF- $\alpha$  production in the urinary bladder tissue. This suggests that leptin treatment protects tissue against I/R-induced injury.

Apoptosis has an important role in maintaining homeostasis in many systems. On the other hand, apoptosis is an active process of cell destruction, important in pathological processes such as bladder I/R injury (25). Oxidative stress is recognized as a strong mediator of apoptosis *via* the formation of lipid hydroperoxides. Lipid hydroperoxides are well known to be highly toxic and induces DNA fragmenta-

tion. Major sources of ROS production takes place in the mitochondrion, endoplasmic reticulum, plasma membrane and cytosol (20). There are many studies demonstrating the anti-apoptotic effects of leptin. The study by Ogunwobi and Beales has indicated that leptin has anti-apoptotic effects on human colon cancer cells (18). In our study, the ratio of TUNEL-positive nuclei in the I-R group was significantly higher than that of both the control and treatment groups although no difference was found between the control and treatment groups. In other words, leptin treatment decreased the ratio of apoptotic cells suggesting that it protects I/R injury.

In conclusion, the results of the biochemical, histological and TUNEL-staining studies have revealed that leptin has protective effects on urinary bladder I/R injury. It plays a role as a potent free-radical scavenger, a powerful antioxidant and it also has protective effects against apoptosis.

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