Original Article

Prophylactic ozone administration reduces renal ischemia-reperfusion injury in the rat

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Abstract: Background: The objective of this study was to examine the role of ozone oxidative preconditioning after renal IR (ischemia reperfusion) injury. Methods: Twenty-eight Wistar rats were randomized into four groups: sham operated (S), IR, ozone (O), and O+IR. The S group was administered physiological saline (PS) intraperitoneally (i.p.) for seven days. The IR group was subjected to renal ischemia for 1 h by occlusion of the left renal artery and vein, followed by reperfusion for 2 h. The O group was administered ozone i.p. for seven days. In the O+IR group, ozone was administered i.p. for seven days before the IR procedure. IR injury (as in the IR group) was induced on the eighth day. Laboratory analyses of renal tissue samples for superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) were performed. Results: The total oxidant score (TOS) and total antioxidant capacity (TAC) of the blood samples were also analyzed. The degree of renal injury was highest in the IR group. In the O+IR group, renal injury was decreased. The antioxidant parameters were increased in the O group. The oxidant parameters were highest in the IR group. Conclusion: Ozone preconditioning ameliorated renal IR injury, with a significant decrease observed in the renal IR injury score.

Keywords: Ischemia reperfusion injury, kidney, ozone, apoptosis

Introduction

Ozone gas was first utilized medically during World War I by the German physician Dr. Albert Wolff in local surgical interventions to prevent wound infections [1]. Since then, it has been used as an antibacterial and antiviral disinfection agent [2]. Clinical studies have investigated the use of ozone in combating ischemia, inflammation, infection, pressure sores, peritonitis, and peripheral vascular diseases in diabetic and nondiabetic patients, stroke patients, and others [3]. Following findings that low doses of ozone gas stimulated antioxidant endogenous systems, ozone therapy has been applied in the treatment of a variety of diseases in experimental and clinical studies. These include cerebrovascular ischemia [4], chronic ulcers [5], arteriosclerosis obliterans [6], immune deficiency [7], hepatic steatosis [8], and heart ischemia [9]. Research has also demonstrated that intravenous ozone treatment decreased blood cholesterol and increased antioxidant responses in cardiomyopathy patients [9]. Ozone is a known modulator of the immune system. In therapy, an oxygen (O\textsubscript{2})/ozone (O\textsubscript{3}) gas mixture is commonly applied, with ozone administered at a rate of around 3% [10]. In human, ozone therapy has been administered via local irrigation, intramuscular, subcutaneous, and intraarticular routes as well as enema [11]. It dissolves in plasma immediately after administration. Ischemia reperfusion (IR)-induced acute kidney injury is linked to an increased risk of acute rejection in kidney transplantation. The incidence of this type of injury in hospitalized patients varies from 2-7\% [12], and it is associated with adverse clinical outcomes and high mortality rates, ranging from 30-70\% [13]. IR-induced acute kidney injury is charac-
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characterized by a complex sequence of events, which is triggered by direct cellular damage caused by hypoxic processes [14]. Ischemic tissue reperfusion aggravates acute ischemic injury via the formation of reactive oxygen and nitrogen components. This is followed by renal dysfunction and damage produced by an inflammatory cascade, resulting in the eventual death of renal cells [14].

To the best of our knowledge, there have been only a few reports of the role of ozone preconditioning in renal IR injury [15-17]. Given the limited number of studies and the effectiveness of ozone treatment in renal IR injury shown in earlier studies, the present study investigated the effect of ozone on renal IR injury by evaluating the total antioxidant capacity (TAC) and total oxidant score (TOS) of the blood and malondialdehyde (MDA), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) levels in renal tissue samples.

Material and methods

The current study was approved by the Selcuk University Animal Ethics Committee and was performed in accordance with the National Institutes of Health guidelines and as recommended by the Science Council of Japan or the National Research Council’s criteria (NIH No. 86-23) for the use of experimental animals. Female Wistar rats weighing 250-300 g were used in this study. The animals were housed under a 12-h light/12-h dark cycle at a temperature of 24±3°C. The animals were fed with a standard pellet diet and water ad libitum.

Experimental groups

The number of rats in the groups was determined according to a previous study [18] and limited to 28 to eliminate excessive sacrifice. The animals were randomly divided into four groups: sham operated (S) (n=7), IR, ozone (O), and O+IR. The S group was given physiological saline (PS) intraperitoneally (i.p.) for seven days. On the eighth day, the right and left renal pedicles were exposed after a midline incision, and laparotomy was performed, but IR injury was not induced. The IR group (n=7) was subjected to renal ischemia for 1 h by occlusion of the left renal artery and vein with a nontraumatic clamp, followed by reperfusion for 2 h and neither PS nor ozone was administered. The O group (n=7) was given ozone at a dosage of 1 mg/kg in a single dose administered i.p. each day for seven days. On the eight day, the right and left renal pedicles were exposed after a midline incision, and laparotomy was performed, but IR injury was not induced. The O+IR group (n=7) was administered 1 mg/kg of ozone i.p. for seven days before the creation of IR injury (as in the IR group) on the eighth day.

Ozone preconditioning

Ozone was generated by an ozone generator machine (Humazon Promedic, Humares® GmbH, Germany), which controls the gas flow rate and ozone concentration in real time using a built-in UV spectrometer. The ozone flow rate was kept constant at 3 L/min in a gas mixture of 97% O₂+3% O₃, representing a concentration of 60 μg/mL. The volume of the gaseous mixture administered to each animal was approximately 3.5-4.5 mL. Tygon polymer tubes and single-use silicon-treated polypropylene syringes (ozone resistant) were used throughout the reaction to ensure containment of ozone and consistency of concentrations. The first dose of ozone was administered seven days before ischemia. In total, seven doses of ozone were administered. The duration of ozone administration and the number of doses were based on previous studies [6, 19].

IR model

The right and left renal pedicles were exposed by a midline incision and laparotomy. The left and right renal pedicles and the arteries were bilaterally occluded using an atraumatic microvascular clamp (Bulldog Artery Clamp; Harvard Apparatus, Holliston, MA, USA) for 60 min. to create total renal ischemia were created. Adequate occlusion was confirmed by a lack of pulsation in the renal pedicles and the presence of pallor in the kidneys. After the ischemic period (60 min), the microvascular clamps were removed, and reperfusion was confirmed by inspecting the color of the kidneys for restoration [20]. Following 2 h of reperfusion, the animals were sacrificed by intracardiac blood-letting. At the time of death, blood was collected by heart puncture for the measurement of the TAC and TOS. Both kidneys were harvested for histopathological evaluation and SOD, GSH-Px, and MDA analyses.
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Histomorphological evaluation of renal tissue

Both kidneys of each animal were removed for histopathological evaluation. The renal tissue samples were fixed in a freshly prepared formalin solution at room temperature and processed for embedding in paraffin. The paraffin-embedded kidney tissues were sectioned into 4-μm thick sections, placed on polylysine-coated slides, and stained with hematoxylin-eosin (H&E). One pathologist blinded to the animal groups evaluated the renal tissue sections exposed to IR by light microscopy. The following were assessed: structural changes in the proximal tubules (tubular atrophy, loss of the tubular brush border, vacuolization, tubular dilatation, cast formation), mononuclear cell (MNC) infiltration, glomerular changes (GCs), total score of histopathological damage (TSHD), and necrotic and apoptotic cells. Cross-sectional images were scored semiquantitatively in terms of tubulointerstitial damage. The scoring system used was 0, absent; 1, mild; 2, intermediate; and 3, severe [20]. The results were observed using a BX-51 Research Microscope System and a DP70 Image Acquisition System (Olympus, Tokyo, Japan). The total histopathological injury score per kidney was calculated by adding all the scores.

To evaluate the apoptosis of the renal tissues at the cellular level, the TUNEL (terminal deoxy-nucleotidyl transferase dUTP nick end) assay and Insitu Cell Death (POD, Roche, Germany) kit were used, according to the manufacturer’s instructions [21]. They were visualized digitally at 40× magnification, and counts were made in at least three different regions.

Biochemical analysis

For the biochemical analysis, the renal tissues were washed twice with a cold saline solution, placed in glass bottles, and stored in a deep freezer at -80°C until processing. The frozen renal tissues were homogenized in ice-cold phosphate buffer (pH 7.4) using a homogenizer (Ultra Turrax IKA T18 Basic, IKA Labortechnic, Staufen, Germany). The homogenates were centrifuged at 14,000 rpm (7,530 g) at 4°C for 10 min, and the supernatant was analyzed. The total protein concentration of the renal tissue homogenates was determined using the method of Lowry et al. [22], with bovine serum albumin as the standard. MDA, GSH-Px, and SOD were measured in the renal tissue samples, and TOS and TAC were measured in the blood samples. In these analyses, 2 mL of intracardiac blood were drawn 2 h after reperfusion. The blood samples were centrifuged at 3,500 rpm for 15 min, and the serum was collected and stored at -80°C until processing.

Measurement of MDA

The lipid peroxidation product and kidney tissues were homogenized in 1.15% KCl solution. A 100 µl aliquot of the homogenate was added to a reaction mixture containing 200 µl 8.1% sodium dodecyl sulfate, 1,500 µl 20% acetic acid (pH 3.5), 1,500 µl 0.8% thiobarbituric acid, and 700 µl distilled water. Samples were then boiled for 1 h at 95°C and centrifuged at 3,000 g for 10 min. The absorbance of supernatant was measured by spectrophotometry at 650 nm. The MDA was assayed using a calorimetric reaction with thiobarbituric acid as described by Shin et al. [23]. The concentration of MDA was expressed as nmol/mg protein.

Measurement of SOD

The activity of SOD was evaluated by inhibition of nitro blue tetrazolium reduction by superoxide anions generated by the xanthine-xanthine oxide system, according to the method of Sun et al. [24]. A commercial assay kit (Nanjing Jiancheng Biological Product, Nanjing China) was used. The calculated SOD activity was expressed as U/g protein.

Measurement of GSH-Px

A Cayman GSH-Px assay kit (Cayman Chemical, USA) was used to measure the activity of GSH-Px. The efficacy of the treatment was assessed by measuring the tissue levels of GSH-Px using the method of Paglia and Valentine [25]. GSH-Px activity is coupled to the oxidation of NADPH by glutathione reductase. The oxidation of NADPH at 37°C was measured using a spectrophotometer. The absorbance at 340 nm was recorded, and GSH-Px activity was recorded as U/g protein in the tissue sample.

Measurement of the TAC

A novel automated measurement method described previously was used to measure the TAC [26]. Using this method, the antioxidative effect of the sample against potent free radical
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Figure 1. The histopathological evaluation markers of the four groups. a: P<0.0001 compared with the 1st group. b: P<0.0001 compared with the 2nd group. c: P<0.0001 compared with the 3rd group. d: P<0.0001 compared with the 4th group.

Figure 2. Apoptotic cell labeling with TUNEL (A) (S Group, DAPI), (B) (S Group, TUNEL): very few apoptotic cells were seen (white arrow heads). (C) (O group, DAPI), (D) (O Group, TUNEL): Slightly higher number of apoptotic cells were seen than S group (white arrow heads).

reactions, which are initiated by the produced hydroxyl radicals, is measured. The TAC was measured using an Aeroset 2.0 analyzer and Cayman's total antioxidant score kit (cayman
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Chemical, USA) according to the manufacturer’s instructions. The results were expressed as μmol Trolox Eq/L. The reaction rate was calibrated with Trolox, which is widely used as a standard in TAC measurement assays.

**Measurement of the TOS**

The serum TOS levels of the blood samples were determined using an automated colorimetric measurement method developed by Erel [27]. The serum TOS levels of the blood samples were determined using an Aeroset 2.0 analyzer and a commercial Cayman’s TOS kit (Cayman Chemical, USA). The assay is based on the oxidation of ferrous ion to ferric ions in the presence of various oxidant species in acidic medium and the measurement of the ferric ions by xylenol orange. The color intensity, which denotes the total amount of oxidant molecules in the sample, was measured spectrophotometrically. The assay was calibrated with hydrogen peroxide (H₂O₂). The results are expressed as micromolar hydrogen peroxide equivalent per liter (μmol H₂O₂ Eq/L).

**Statistical analysis**

Statistical analysis was performed using SPSS 15.0 for Windows (SPSS Inc., IL, USA). The results are expressed as mean ± standard deviation. The differences in pathological findings between the study groups were analyzed using the Kruskal-Wallis test. When an overall statistically significant difference was observed, pairwise comparisons were performed using the Mann-Whitney U test. Results were considered statistically significant if the two-tailed P was <0.05.

**Results**

**Histopathological results (Figures 1-4)**

Apoptotic index (AI): The AI was significantly different between the four groups (P trend
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<0.0001) (Table 1). In the pairwise analysis, the AI was higher in the IR group than in the O+IR (P=0.002) and higher in the S (P=0.002) group than in the O (P=0.002). The AI was higher in the O+IR group than in the S (P=0.002) and O groups (P=0.002). The AI was higher in the S group than in the O group (P=0.002).

Mononuclear cell infiltration (MCI): The MCI was significantly different between the four groups (P trend <0.0001) (Table 1). In the pairwise analysis, the MCI was higher in the IR group than in the O+IR group (P=0.002) and higher in the S (P=0.002) group than in the O group. The MCI was higher in O+IR group than in the S group, but the difference was not statistically significant (P=0.606) compared to that of the O group (P=0.002). The MCI was higher in the S group than in the O group, but the difference was not statistically different (P=0.591).

Proximal tubular changes (PTC): The PTC was significantly different between the four groups...
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Table 1. The histopathological evaluation markers of the four groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>(S) (N=7)</th>
<th>(IR) (N=7)</th>
<th>(O) (N=7)</th>
<th>(O+IR) (N=7)</th>
<th>PTtrd</th>
</tr>
</thead>
<tbody>
<tr>
<td>AI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTC</td>
<td></td>
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<tr>
<td>GC</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>TSHD</td>
<td></td>
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</tbody>
</table>

(P trend <0.0001) (Table 1). In the pairwise analysis, the PTC was higher in the IR group than in the O+IR group (P=0.008) and higher in the O group than the S group, but the difference was not statistically significant (P=1.00). The PTC was higher in the O+IR group than in the S (P=0.002) and O groups, but the difference was not statistically significant (P=1.00).

Glomerular changes (GCs): The GCs were significantly different between the four groups (P trend <0.0001) (Table 1). In the pairwise analysis, the level of GCs was greater in the IR group than in the O+IR (P=0.000) group and greater in the O (P=0.000) group than in the S group (P=0.000).

Total score of histopathological damage (TSHD): TSHD was significantly different between the four groups (P trend <0.0001) (Table 1). In the pairwise analysis, TSHD was higher in the IR group than in the O+IR (P=0.001) group and higher in the S group (P=0.000) than in the O group (P=0.000). TSHD was higher in the IR group than in the O+IR group (P=0.000).

Table 2. The biochemical markers of the four groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>(S) (N=7)</th>
<th>IR (N=7)</th>
<th>O (N=7)</th>
<th>O+IR (N=7)</th>
<th>PTtrd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase</td>
<td>1.26±0.06bcd</td>
<td>0.91±0.12abc</td>
<td>1.64±0.03abcd</td>
<td>1.46±0.01abcd</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>1.57±0.4bcd</td>
<td>1.59±0.03abcd</td>
<td>2.50±0.03abcd</td>
<td>2.11±0.02abcd</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Malondialdehyde</td>
<td>6.06±0.09abcd</td>
<td>7.39±0.22abcd</td>
<td>4.50±0.31abcd</td>
<td>5.44±0.14abcd</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total antioxidant capacity</td>
<td>2.50±0.04abcd</td>
<td>1.84±0.03abcd</td>
<td>4.58±0.34abcd</td>
<td>3.52±0.19abcd</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total oxidant status</td>
<td>8.35±0.36abcd</td>
<td>14.27±0.63abcd</td>
<td>4.93±0.53abcd</td>
<td>7.35±0.54abcd</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

(P trend <0.0001) (Table 1). In the pairwise analysis, the PTC was higher in the IR group than in the O+IR (P=0.008) and higher in the O group than the S group, but the difference was not statistically significant (P=1.00). The PTC was higher in the O+IR group than in the S (P=0.002) and O groups, but the difference was not statistically significant (P=1.00).

Glomerular changes (GCs): The GCs were significantly different between the four groups (P trend <0.0001) (Table 1). In the pairwise analysis, the level of GCs was greater in the IR group than in the O+IR (P=0.000) group and greater in the O (P=0.000) group than in the S group (P=0.000). The GCs were significantly different between the four groups (P trend <0.0001) (Table 1). In the pairwise analysis, the level of GCs was greater in the IR group than in the O+IR (P=0.000) group and greater in the O (P=0.000) group than in the S group (P=0.000).

Total score of histopathological damage (TSHD): TSHD was significantly different between the four groups (P trend <0.0001) (Table 1). In the pairwise analysis, TSHD was higher in the IR group than in the O+IR (P=0.001) group and higher in the S group (P=0.000) than in the O group (P=0.000). TSHD was higher in the IR group than in the O+IR group (P=0.000).
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Biochemical results (Figure 5)

SOD: The level of SOD was significantly different between the four groups (P trend: 0.001) (Table 2). In the pairwise comparison, the level of SOD was significantly higher in the O group than in the S group, and higher in the IR group than in the O+IR group (P for all 0.001). The level of SOD was significantly higher in the O+IR group than in the S and IR groups (P for all 0.001). The level of SOD was also significantly higher in the S group than in the IR group (P=0.001).

GSH-Px: The level of GSH-Px was significantly different between the four groups (P trend < 0.0001) (Table 2). In the pairwise comparison, the level of GSH-Px was significantly higher in the O group than in the S group and higher in the IR group than in the O+IR group (P for all 0.001). The level of GSH-Px was significantly higher in the O+IR group than in the S and IR groups (P for all 0.001). The level of GSH-Px was not significantly different between the S and IR groups (P=0.445).

MDA: The level of MDA was significantly different between the four groups (P trend < 0.0001) (Table 2). In the pairwise comparison, the level of MDA was significantly higher in the IR group than in the S, O and O+IR groups (P for all, 0.001). The level was also higher in the S group than in O and O+IR groups (P for all, 0.001). The level of MDA was higher in the O+IR group than in the O group (P=0.001).

TAC: The TAC was significantly different between the four groups (P trend <0.0001) (Table 2). In the pairwise comparison, the TAC was significantly higher in the O group than in the S, IR, and O+IR groups (P for all, 0.001). The TAC was significantly lower in the IR group than in the S and O+IR groups (P=0.001 and 0.001, respectively). The TAC was also lower in the S group than in the O+IR group (P=0.001).

TOS: The TOS was significantly different between the four groups (P trend <0.0001) (Table 2). In the pairwise comparison, the TOS was significantly higher in the IR group than in the S, O and O+IR groups (P for all, 0.001). The TOS was also higher in the S group than in the O and O+IR groups (P for all, 0.001). The TOS was higher in the O+IR group than in the O group (P=0.001).

Discussion

Renal IR injury is a common cause of acute renal injury, and it is characterized by high mortality and morbidity [28]. Although there have been many studies of renal IR injury [29, 30], a therapeutic strategy for renal IR has not been developed until recently. Oxidative stress is the main mechanism underlying IR injury [31]. It results in free oxygen radicals attacking the reperfused tissue. The binding of these radicals to lipids, proteins, and nucleic acids on cell membranes causes lipid peroxidation, leading to tissue damage [31]. The expression of adhesion molecules in response to vascular dysfunction results in transmigration of polymorphonuclear leukocytes into renal tissues and immune system activation, especially of T cells and cell adhesion molecules [32]. The subsequent production of reactive oxygen species (ROS) and nitric oxide contribute to the destruction and dysfunction of renal tissue [32]. Low doses of ozone can promote oxidative preconditioning by enhancing the activity antioxidant endogenous systems, thereby contributing to the preservation of the redox state of the cell [5]. A previous study demonstrated that ozone supports cellular antioxidant systems and prevents septic shock by mediating the expression of ROS/RNS [33]. According to the results of the present study, ozone used alone or in combination significantly reduced MNC infiltration, glomerulotubular changes, and total damage scores in IR injury. The significant decrease in apoptosis in the O+IR group suggested that could be useful in the treatment of IR.

In the present study, lipid peroxidation, which is an oxidative stress marker of MDA, was increased in the kidneys of the IR group. Levels of the antioxidant enzymes SOD and GSH-Px were elevated in the O group. These results are similar to those of previous studies, which reported that ozone prevented apoptosis and exerted an anti-inflammatory effect in renal IR injury [33] and that ozone decreased long-term renal fibrosis in IR injury [34]. Additionally, the present study revealed statistically significant changes in the plasma TAC and

Table 2

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TOS levels after both IR and ozone exposure. The significant elevation in the plasma TOS level in the IR groups may be related to an increase in renal oxidant stress after the IR injury. The plasma TAC of the animals treated with ozone was remarkably increased, which suggests augmentation of renal antioxidant defenses. A previous study also reported a significant reduction in the serum TAC level of an IR group, in addition to increases in plasma MDA concentrations [35].

The results of the present study indicated that ozone pretreatment activated renal antioxidant mechanisms, which may be involved in conferring renal protection against IR injury. The pretreatment with ozone before IR injury decreased oxidative injury, as demonstrated by significantly reduced serum MDA concentrations in the O group. After the administration of ozone, it immediately dissolves in plasma and reacts with macromolecular glycoproteins, such as carbohydrates and polypeptides. The oxidation of these compounds results in the formation of H$_2$O$_2$ and lipid oxidation products, which act as electron donors. H$_2$O$_2$ acts immediately and disappears (early and short-acting messenger) and is able to act as an oxygen messenger, which acts as a regulator of signal transduction and is an important mediator of host defense and immune responses [5]. In contrast to H$_2$O$_2$, lipid oxidation products are distributed throughout the tissues, and they have long-lasting effects, stimulating the innate immune system and aiding cell survival in response to IR injury [36]. The beneficial actions of ozone have been demonstrated previously, with the gas used as a therapeutic agent in experimental and clinical studies [2, 3, 6, 10]. Our previous in vivo study showed that ozone attenuated intestinal IR injury [19]. We also showed that ozone ameliorated eye IR injury, cochlea IR injury, and flap IR injury (unpublished data).

Renal IR injury associated with renal transplantation is an unresolved problem in clinical practice. The experimental model used in this work mimicked the IR injury that occurs during renal transplantation procedures. The model can be used to shed light on complex processes of acute renal damage that cannot be studied in humans due to ethical concerns. The mechanisms underlying the positive effects of ozone are not fully understood. However, various mechanisms have been proposed [36]. Among these, the most important is up-regulation of cellular antioxidant enzyme activity and regulation of ROS [37], which protects the host against pathophysiological conditions mediated by ROS.

In the current study, the application of ozone i.p. for seven days had a beneficial effect on both antioxidant and histopathological values in renal hypoxic/reperfusion injury. In common with the findings of an earlier study by Oztosun et al. [15], ozone protected renal cells and lifetime against IR injury. In contrast to the study by Oztosun et al., TAC, which is an antioxidant marker, and TOS, which is an oxidative marker, were evaluated in the present study. In addition, the levels of the antioxidants SOD and GSH-Px and those of the oxidant MDA in kidney tissue were evaluated. The results of these analyses corroborated the histopathological findings and the results of other biochemical tests.

In this study, lipid peroxidation, which is an oxidative stress marker for MDA, was increased in the kidneys of the IR-induced rats. The activities of the antioxidant enzymes SOD and GSH-Px were also increased in the ozone treated groups. These results are similar to those reported in previous studies of IR injury in other organs, such as liver [2], heart [6], soft tissue [38], intestines [19], and rat skeletal muscle [39]. Ozone was also shown to protect against IR injury in a testicular torsion model [40] and ovary torsion model [10]. The results of the present study are consistent with those of the above studies, with ozone providing protection against renal IR injury. In clinical use, particularly in liver, kidney, eye, and composite tissue transplantation, the administration of ozone prior to transplantation may provide protection from IR injury, which occurs frequently in such procedures. However, the use of ozone in humans remains controversial. Therefore, its clinical use is restricted. Further studies of the preventive and protective effects of ozone in patients with IR injury are required.

The most important limitation of the present study is that the ozone was administered before the creation of the IR injury. However, considering planned organ transplantations, this may not be seen as a limitation. Nevertheless,
administering ozone both before and after IR injury would yield more reliable results. To shed light on this issue, we administered ozone before and after IR in a study of the effect of ozone on flap viability (unpublished data). The results demonstrated the healing effect of ozone on IR injury. A second limitation of this study is that the animals were not housed in metabolic cages. Thus, this study lacks data on other well-documented renal injury biomarkers, such as creatinine clearance and urine microprotein.

**Conclusion**

Various studies have shown that ozone therapy is beneficial for the treatment of different clinical entities [2, 3, 6, 10, 11]. The findings of the present study suggest that ozone may provide renal protection against IR injury by decreasing tissue damage and increasing the activity of antioxidant endogenous systems in a rat model. Further studies are required to explain the mechanisms mediating the protective effect of ozone on renal IR injury.

**Disclosure of conflict of interest**

None.

**Abbreviations**

S, sham operated group; TAC, total antioxidant capacity; IR, ischemia reperfusion; PS, physiological saline; O, O$_3$, ozone; MNC, mononuclear cell; O+IR, ozone+ischemia reperfusion group; GCs, glomerular changes; SOD, superoxide dismutase; TSHD, total score of histopathological damage; GSH-Px, glutathione peroxidase; MDA, Malondialdehyde; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end; H$_2$O$_2$, hydrogen peroxide; TOS, total oxidant score; AI, Apoptotic index; PTC, Proximal tubuler changes; GC, Glomeruler changes.

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