The preventive role of medical ozone therapy on remote organ injury induced by hepatic ischemia reperfusion

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Abstract

Aim: This study explores the effects of ozone therapy on the prevention of remote organ injury in rats with induced hepatic ischemia-reperfusion injury.

Materials and Methods: Twenty-one male Sprague Dawley rats were sorted randomly into three groups: (a) A control group in which the rats' abdomens were operated on by laparotomy, and their livers then viewed and closed with a suture; (b) Rats with induced hepatic ischemia-reperfusion injuries; and (c) Rats induced with the same injuries that were administered ozone therapy before reperfusion. The rats' hepatic arteries, portal veins, and bile ducts were clamped, and ischemia was induced for 45 min. Reperfusion was provided for 90 min in the second and third groups. The third group was administered 0.5 mg/kg ozone intraperitoneally before reperfusion. Liver, lung, and kidney tissues were taken for biochemical and histopathological examinations.

Results: In the second group, malondialdehyde levels in the lung and kidney tissues were higher than in the control group, while catalase and superoxide dismutase enzyme levels were lower. The third group had lower malondialdehyde levels and higher catalase and superoxide dismutase enzyme levels than the group that did not receive the therapy. In histopathological evaluations, the damage to both kidney and lung tissues in the third group was higher than in the control group; however, this damage was significantly lower than in the second group.

Conclusions: The findings indicated that the administration of ozone therapy attenuated lung and kidney injuries caused by hepatic ischemia, since partial protection was demonstrated in many instances.

Keywords: Hepatic ischemia/reperfusion; kidney; lung; ozone; remote organ injury

INTRODUCTION

Significant levels of cell damage and organ function disorder have been known to occur when tissues or organs have been exposed to ischemia and subsequent reperfusion (1). Hepatic ischemia-reperfusion (IR) injury may occur following liver transplantation or resection, veno-occlusive disease, heart failure, and cardiogenic or hemorrhagic shock (2-4). It may cause liver dysfunction or failure (2) as well as significant injuries to other organs, including the lungs and kidneys (5,6). Reactive oxygen species (ROS) generally play a triggering role in IR injuries, including hepatic IR. Acute ROS production occurring subsequent to the reoxygenation of the liver initiates deleterious cellular responses chain that results in tissue injuries, inflammation, cell death, and organ failure (7).

Ozone (O3) is a gas molecule with a cyclic structure composed of three oxygen atoms. It acts as a strong disinfectant and causes damage to the respiratory system and extra-pulmonary organs when inhaled. However, several recent studies have indicated that ozone has a curative effect when it is administered in appropriate doses and when the length of exposure is adjusted properly (8,9). Ozone therapy is administered as an oxygen–ozone mixture. Previous studies have investigated the protective effects of ozone therapy on various liver injury models, finding that non-toxic doses of ozone provided adaptation capability against oxidative stress. This adaptability was provided by its ability to shift the balance between endogenous antioxidants and pro-oxidants to favor the former (10,11).

In this study, we investigated the effects of ozone therapy on remote tissue injury in the lungs and kidneys of rats with induced hepatic IR injuries using biochemical parameters and histopathological examination.

MATERIALS and METHODS

Chemicals
Ketamine hydrochloride was obtained from Pfizer (Ketalar, Istanbul, Turkey), xylazine hydrochloride from Bayer (Rompun, İstanbul, Turkey), and sodium thiopental from İE Ulagay (Pental Sodium, Istanbul, Turkey). All other chemicals were purchased from Sigma Chemical (St. Louis, Missouri, USA) and MERCK (Darmstadt, Hesse, Germany). The oxygen−ozone mixture (95% oxygen and 5% ozone) used in the medical ozone therapy was created using an ozone generator device manufactured by Herrmann Apparatebau GmbH (Kleinwallstadt, Bavaria, Germany).

Animals
Twenty-one male Sprague Dawley rats, each weighing 220−240 g, were procured from Ataturk University's Medical Practice and Research Center (Erzurum, Turkey). The care and application process was carried out in accordance with National Laboratory Animals Ethical Rules. The study was conducted after obtaining the approval of Atatürk University's Animal Experiments Local Ethics Committee (Reference No: 36643897−112−92). The rats were housed in plastic breeding cages, which were kept at a constant temperature of 22 ± 1 °C. They were exposed to 12 hr of daylight and 12 hr of dark each day. Pellet chow and tap water were provided ad libitum for their standard feeding.

Surgical Procedures and Experimental Groups
Before starting the animal applications, the rats were anesthetized using a mixture 30 mg/kg ketamine and 6 mg/kg xylazine administered intraperitoneally. Anesthetic doses were readministered when signs of recovery were observed. The rats were randomly sorted into three groups of seven:

a.Control group (C)
The abdomen of each rat in this group was opened with abdominalization and, after visualization of the liver, closed with a weak suture.

b.Hepatic ischemia-reperfusion group (IR)
The abdomen of each rat was opened with abdominalization. The hepatic artery, portal vein, and bile duct were clamped with a vascular clamp. After a 45−min period of ischemia, the clamp was removed and a 90−min period of reperfusion provided.

c.Hepatic ischemia-reperfusion with ozone therapy group (IR+O)
As in the IR group, a 45−min period of ischemia was induced in the rats followed by a 90-min period of reperfusion. 0.5 mg/kg of ozone (25 µg/ml in saline, 0.9% NaCl) of ozone was administered intraperitoneally immediately preceding reperfusion.

After these processes were complete, the rats were euthanized with a 120 mg/kg dose of sodium thiopental administered intraperitoneally. Liver, lung, and kidney tissue samples were then taken from the rats. One piece of each sample was placed in a 10% buffered formalin solution for histopathological examination. Another piece of each sample was stored at −80 °C for biochemical analysis.

Biochemical Procedures
The tissues taken from the rats were homogenized with a 0.2 mM Tris-HCl pH 7.4 buffer (IKA Ultra−Turrax T 25 basic homogenizer, Germany), and tissue malondialdehyde (MDA) levels were measured. The homogenate was then centrifuged at 4000 rpm for 55 min, and the supernatant was separated for catalase (CAT), glutathione reductase (GR), and superoxide dismutase (SOD) measurements. For the SOD measurement, the supernatant was extracted with an equal volume ethanol−chloroform (5/3, v/v) mixture. The spectrophotometric measurements were performed with a Shimadzu UV-1600 spectrometer (Kyoto, Japan).

The MDA level was measured by its reaction with thiobarbituric acid (TBA), which produces a pink color with absorption at 532 nm. The reaction was performed in acidic medium (pH = 29) at 90−95 °C (12). The results were calculated according to the standard curve, and measured in nM/g of wet tissue.

CAT (EC 1.11.1.6) enzyme activity was spectrophotometrically measured at 240 nm according to the principle of hydrogen peroxide's (H_2O_2) consumption by the catalase in the medium (13). Hydrogen peroxide was added to a 50−mM phosphate buffer, and the buffer was adjusted to 0.500 optical densities (OD). As the sample was supplemented, the decrease in hydrogen peroxide concentration was measured and recorded at 15−s intervals. The rate of hydrogen peroxide consumed in 1-min was expressed as a k/g protein (k = [2.3 × log (OD1/ OD2)]/30 s).

GR (EC 1.8.1.7) enzyme activity was measured by the reduction of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase at 340 nm. One enzyme unit was considered as the reduction of 1 mM NADPH in 1 min (25 °C, pH 8.0) (14). GR enzyme activity was expressed as U/mg of protein.

SOD (EC 1.15.1.1) enzyme activity was measured at 560 nm based on the principle of reduction of nitro blue tetrazolium (NBT) by O2 (15). It became lighter in color in accordance with the amount of SOD enzyme present, while the color became darker when the enzyme was absent. One unit of SOD was adopted as the amount of enzyme required to inhibit NBT reduction at a rate of 50%, expressed as U/mg of protein (enzyme inhibition % = ($Abs_{control}$ − Abs_{sample}) / $Abs_{control}$ × 100).

The protein analyses of the extracted samples and supernatants were carried out according to the method used previously by Lowry et al (16).

Histopathological Evaluation

Tissue samples were taken from the rats following euthanasia and fixed in a 10% neutral buffered formalin solution for 2 days. Subsequently, the samples were washed under flowing water, dehydrated with ethanol, and placed in xylene for clearing. The samples were then embedded in paraffin and cut into 5−µm−thick slides using a Leica RM2235 microtome (Leica Instruments, Nussloch, Germany), which were then stained with hematoxylin and eosin. Four scores were used for histopathological evaluation: (0) none, (1) mild, (2) moderate, and (3) severe (17,18). The semi-quantitative evaluation was performed by a pathologist who was blinded to the study groups and their biochemical results. Slides were evaluated with a light microscope (Olympus BX53, Tokyo, Japan) supported with a digital camera and an imaging system (CellSen, Olympus, Tokyo, Japan).

Statistical Analysis

The statistical analysis of the numerical data was carried out using IBM SPSS 17 with Microsoft Windows 10. The value of $p < 0.05$ was accepted as statistically significant. The results were expressed as the mean value ± standard deviation. The nonparametric Kruskal−Wallis test was used to compare the data obtained from all groups, and paired comparisons between groups were evaluated with the Mann−Whitney U test.

RESULTS

MDA, CAT, GR, and SOD levels (Table 1) were compared across groups. Significant differences were observed between all groups in the MDA, CAT, and SOD levels in lung and kidney tissue samples.

MDA, CAT, and SOD levels in both lung and kidney tissues progressed similarly. The IR group's MDA level was significantly higher than that of the C group, and the MDA level in the IR+O group was significantly lower than in the IR group. The difference between the C group and the IR+O group was insignificant ($p > 0.05$).

The CAT and SOD levels in the IR group were significantly lower than in the C group. The IR+O group's CAT level was significantly higher than that observed in the IR group; the SOD level was higher in both lung and kidney tissues, although this difference was only statistically significant in the kidney tissues.

Histopathological evaluations are presented in Table 2. While the scores of (0), (1), and (2) were noted in the tissues, a score of (3) was not observed in any of them (Figure 1). Intergroup comparisons made for lung and kidney tissue samples were statistically significant. While no injury was observed in the lung and kidney tissues of rats in the C group, a severe level of tissue injury was observed in the IR group. Tissue injury in the IR+O group was significantly lower than in the IR group; however, it was significantly higher compared to the C group.

| Table 1. The biochemical values of malondialdehyde (MDA), catalase (CAT), glutathione reductase (GR), and superoxide dismutase (SOD) in the kidney and lung tissue samples, given as the mean value ± standard deviation and median (min-max)				
Categorical variables	MDA	CAT	GR	SOD
Kidney				
C (n = 7)	2.876±0.528	0.692±0.075	0.007±0.001	0.020±0.003
	3.145 (2.014-3.516)	0.687 (0.614-0.806)	0.006 (0.005-0.008)	0.020 (0.017-0.025)
IR (n = 7)	4.040±0.698[a]	0.523±0.064[c]	0.007±0.001	0.014±0.003[c]
	3.956 (3.318-5.186)	0.547 (0.417-0.589)	0.007 (0.005-0.009)	0.014 (0.008-0.018)
IR+O (n = 7)	3.145±0.412[d]	0.625±0.062[b]	0.007±0.001	0.018±0.003[b]
	3.017 (2.813-3.998)	0.613 (0.556-0.749)	0.006 (0.005-0.008)	0.018 (0.012-.0.021)
Lung				
C (n = 7)	0.447±0.133	0.059±0.009	0.005±0.001	0.007±0.001
	0.417 (0.305-0.660)	0.058 (0.044-0.071)	0.005 (0.004-0.006)	0.007 (0.005-0.008)
IR (n = 7)	0.673±0.115[a]	0.045±0.008[c]	0.006±0.001	0.005±0.001[c]
	0.672 (0.524-0.856)	0.042 (0.038-0.056)	0.006 (0.005-0.007)	0.005 (0.005-0.006)
IR+O (n = 7)	0.461±0.083[d]	0.055±0.006[b]	0.006±0.001	0.006±0.001
	0.412 (0.385-0.583)	0.054 (0.046-0.064)	0.006 (0.005-0.007)	0.005 (0.005-0.006)
$p < 0.05$ statistically significant increase, (a) when compared with the control (C) group, (b) when compared with the ischemia-reperfusion (IR) group. $p < 0.05$ statistically significant decrease, (c) when compared with the C group, (d) when compared with the IR group				

Table 2. The tissue damage scores of kidneys and lungs given as the mean value ± standard deviation					
Categorical variables	Histopathological scores				Mean±SD
	0	I	II	III	
Kidney					
C (n = 7)	7				0.000 ±0.000
IR (n = 7)			2	5	1.710±0.488[a]
IR+O (n = 7)	2	5			0.710±0.488[b]
Lung					
C (n = 7)	7				0.000 ±0.000
IR (n = 7)				7	2.000±0.000[a]
IR+O (n = 7)	1	5	1		1.000±0.577[a,b]

(a), p < 0.05 statistically significant increase compared with control (c) group. (b), p < 0.05 statistically significant decrease compared with the ischemia-reperfusion (IR) group
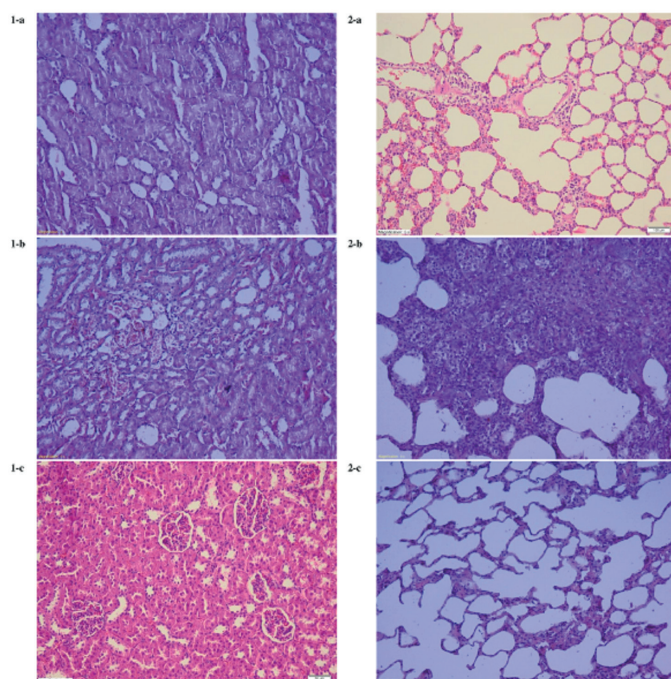
(1-a) the normal histological appearance (Control group), (1-b) the loss of tubular epithelial cells and congestion in the interstitial area (IR group), (1-c) the minimal loss of tubular epithelial cells and congestion (IR+O group). Histopathological images of lung tissues: (2-a) the normal histological appearance (Control group), (2-b) Polymorphonuclear leukocyte and macrophage infiltration in the interstitial area and deterioration of the alveolar structure (IR group), (2-c) slight congestion in the interstitial field and mild polymorphonuclear leukocyte and macrophage infiltration in the interstitial area (IR+O group)

Figure 1. Histopathological images of kidney tissues

DISCUSSION

There have been several studies carried out on hepatic IR injury and the preventive effect of ozone therapy on hepatic IR injury. The basic hypothesis inferred from the data obtained in previous studies was that ozone therapy has a preventive role against the oxidative stress resulting from liver IR (11,19). These results were also obtained by our study (data not shown); however, there has been no study investigating the effect of ozone therapy on remote organ injury prior to our own. In this study, we focused on the possible antioxidative effects of ozone therapy on remote organ injury resulting from the oxidative stress caused by hepatic IR.

MDA is a natural result of lipid peroxidation that causes mutagenic and carcinogenic effects (20). It is one of the most important indicators of lipid peroxidation (21). Previous studies have used increased MDA levels as an indicator of IR injury formation and remote organ injury induced by IR injury (22,23). In their study of hepatic IR injuries induced in rats, Chan et al. (5) accepted that an increase to MDA levels in lung tissue was an indicator of oxidative stress, finding that propofol decreased MDA levels. In another study of hepatic IR injury induced in rats, the MDA level in kidney tissue increased (24). In our study, the MDA level in kidney and lung tissue samples was significantly higher in the IR group than in the C group. Lower MDA levels in the IR+O group indicate that ozone therapy decreases MDA level in the IR group. These findings are similar to those of other studies that investigated the reparative effect of ozone therapy on different IR injury models (25,26).

Oxidative stress refers to the imbalance between reactive oxygen and nitrogen species, namely oxidants and the antioxidants (27). When any stress occurs, decreasing antioxidative enzyme levels are a predictable result. The CAT, GR, and SOD enzymes tested in our study clearly played an important role against oxidative stress (28,29). Although the change in GR enzyme level was not significant, CAT and SOD levels were lower in the IR group than in the C group when both kidney and lung tissues were considered in our study. The various biochemical parameters used in previous studies on hepatic IR remote organ injury models progressed in the same way (5, 6,30). In IR injury models created in many organs including the liver; antioxidative enzymes, especially CAT and SOD, were found to be significantly reduced in the inducted groups compared to the control groups (31-34). Tufek et al. (35) found that total oxidative activity and oxidative stress index values increased in intergroup comparisons made between sham and hepatic IR groups in serum, liver, lung, and kidney samples. In the same study, the decrease of total antioxidant capacity and para-oxonase values in the IR group indicated the formation of antioxidant stress in organs such as the liver, lungs, and kidneys as a result of hepatic IR. When compared to the IR group, the increase of enzyme levels in the IR+O group indicated that ozone decreased injury in both kidney and lung tissues. This demonstrates that ozone is effective at removing and eliminating oxidative stress resulting from hepatic IR remote organ injury models.

Our histopathological data are compatible with the results obtained from the biochemical parameters. When data obtained from both lung and kidney tissues were compared to the control, it was determined that serious tissue injury occurred in the IR group and this tissue injury was significantly decreased when ozone therapy was

administered. Our results are similar to the data obtained in previous studies (36,37). When the literature was reviewed, no findings indicating a positive effect of ozone therapy on remote organ injury induced by hepatic IR were noted; however, a positive effect of ozone on hepatic IR models had previously been suggested (11,19,38).

CONCLUSION

In light of our findings, it is possible to conclude that remote organ injury in rats with induced hepatic IR injuries occurs at a significant level and that ozone therapy has a protective and prophylactic effect on preventing this injury. This remote tissue injury was probably induced by the oxygen free radicals that appeared because of liver reoxygenation. Moreover, although ozone had toxic effects, ozone could provide its protective and prophylactic properties revealed in our study through increasing the antioxidant endogenic systems. Considering that ozone had oxidative properties, it was reported that this substance activated specific cell sensors via lipid oxidation products, thus upregulating the antioxidant system at moderate doses (10). Accordingly, ozone show adaptation ability against both itself and other physiopathological alterations mediated by reactive oxygen species (ROS). Consequently, future studies should be performed to evaluate the protective effects of ozone therapy on clinical applications such as liver transplantation in which remote tissue injury occurs.

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