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Original Article / *Özgün Makale*

The effect of ozone treatment on remote organ myocardial injury in an aortic ischemia-reperfusion model

Aortik iskemi reperfüzyon modelinde ozon tedavisinin uzak organ miyokardiyal hasar üzerine etkisi

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ABSTRACT

Background: This study aims to investigate the effect of ozone on myocardial ischemia-reperfusion injury occurring after occlusion - reperfusion of infrarenal abdominal aorta in rats.

Methods: Thirty-two Wistar albino rats (weighing 200-250 g) were randomized into four equal groups. The control (sham) group underwent laparotomy and dissection of the infrarenal abdominal aorta without occlusion. Intraperitoneal ozone was applied for 10 days 1 mg/kg/day in the control+ozone group. Afterwards, control+ozone group underwent laparotomy and dissection of the infrarenal abdominal aorta without occlusion. Aortic ischemia-reperfusion and aortic ischemia-reperfusion+ozone groups underwent dissection of the infrarenal abdominal aorta, followed by achieving ischemia and reperfusion by cross-clamping the infrarenal abdominal aorta for 60 minutes and removing the cross-clamp for 60 minutes, respectively. The tissue levels of malondialdehyde and activity levels of superoxide dismutase, catalase, and myeloperoxidase were measured in the myocardial specimens. The tumor necrosis factor, interleukin-6 and troponin-I levels were measured in the plasma. A histopathological examination of the myocardial specimens was undertaken.

Results: Biochemical analysis showed that aortic ischemia-reperfusion significantly increased ($p<0.05$ vs. control) while ozone significantly decreased ($p<0.05$ vs. aortic ischemia-reperfusion) the myocardial tissue levels of superoxide dismutase and catalase and level of plasma troponin-I. Histologically, in the aortic ischemia-reperfusion group, myocardial disorganization, myofiber swelling and myofiber eosinophilia in the myocardial tissue samples were significantly increased compared to the control group ($p<0.05$ vs. control). However, histopathological changes in the aortic ischemia-reperfusion+ozone group decreased compared to the aortic ischemia-reperfusion group.

Conclusion: The results of this experimental study indicate that ozone attenuates myocardial injury and oxidative stress that develop after infrarenal aortic ischemia-reperfusion through three markers; *(i)* decreased tissue superoxide dismutase and catalase levels, *(ii)* decreased plasma troponin-I levels, and *(iii)* reduced histopathological changes, albeit not statistically significant.

Keywords: Ischemia; myocardial injury; ozone; reperfusion.

ÖZ

Amaç: Bu çalışmada sıçanlarda infrarenal abdominal aortun oklüzyonu-reperfüzyonu sonrası miyokardiyal iskemi reperfüzyon hasarına ozonun etkisi araştırıldı.

Çalışma planı: Otuz iki Wistar albino sıçan (200-250 g ağırlığında) dört eşit gruba randomize edildi. Kontrol (sham) grubunda laparotomi ve oklüzyon olmaksızın infrarenal abdominal aort diseksiyonu uygulandı. Kontrol+ozon grubunda 10 gün 1 mg/kg/gün intraperitoneal ozon uygulandı. Daha sonra, kontrol+ozon grubunda laparotomi ve oklüzyon olmaksızın infrarenal abdominal aort diseksiyonu uygulandı. Aortik iskemi reperfüzyon ve aortik iskemi reperfüzyon+ozon gruplarında infrarenal abdominal aort diseksiyonu uygulandı, takiben infrarenal abdominal aorta 60 dakika kros-klemp konularak ve 60 dakika kros-klemp kaldırılarak sırasıyla iskemi ve reperfüzyon gerçekleştirildi. Miyokardiyal örneklerde dokulardaki malondialdehit düzeyleri ve süperoksit dismutaz, katalaz ve myeloperoksidaz aktivite düzeyleri ölçüldü. Plazma tümör nekroz faktörü, interlökin-6 ve troponin-I düzeyleri ölçüldü. Miyokardiyal örneklerin histopatolojik incelemesi yapıldı.

Bulgular: Biyokimyasal analiz; ozonun doku süperoksit dismutaz ve katalaz düzeylerini ve plazma troponin-I düzeyini anlamlı olarak azaltırken (aortik iskemi reperfüzyona karşı $p<0.05$) aortik iskemi reperfüzyonun anlamlı olarak artırdığını gösterdi (kontrole karşı $p<0.05$). Histopatolojik olarak aortik iskemi reperfüzyon grubundaki miyokardiyal doku örneklerinde miyokardiyal disorganizasyon, myofibriler şişme ve myofibriler eozinofili kontrol grubuna kıyasla anlamlı olarak arttı (kontrole karşı $p<0.05$). Bununla birlikte, aortik İR+ozon grubunda histopatolojik değişiklikler aortik iskemi reperfüzyon grubuna göre azaldı.

Sonuç: Bu deneysel çalışmanın sonuçları ozonun infrarenal aortik iskemi reperfüzyon sonrası oluşan miyokardiyal hasarı ve oksidatif stresi üç temel belirteçle azaltabileceğini gösterdi: *(i)* azalmış doku süperoksit dismutaz ve katalaz düzeyleri, *(ii)* azalmış plazma troponin-I düzeyleri ve *(iii)* istatistiksel olarak anlamlı olmamakla beraber azalmış histopatolojik değişiklikler.

Anahtar sözcükler: İskemi; miyokardiyal hasar; ozon; reperfüzyon.

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Cross-clamping technique performed during infrarenal abdominal aorta (IAA) surgery and the post-surgical removal of the cross-clamp may lead to aortic ischemia-reperfusion (IR) injury.[1] Aortic IR injury causes the formation of free oxygen radicals, systemic vasoconstrictive mediators, neutrophil activation, lipid peroxidation, and systemic inflammatory response, which lead to the development of distant organ damage.[1,2] Therefore, myocardial injury induced by aortic IR is considered as an important complication in the development of high-mortality heart failure after aortic surgery.

In systemic application, ozone (O_3) is known to activate red blood cells and immunocompetent cells, induce biological antioxidants, and remove free radicals.[3,4] In medical and clinical applications, O_3 is mostly acknowledged with strong antifungal, antiviral and antibacterial properties.[4,5] For clinical and medical use, O_3 needs to be prepared as an O_2/O_3 mixture since the reaction of ozone with air may result in the formation of nitrogen dioxide, a toxic gas.[6] Since O_3 is acquired by the disintegration of O_2 and has an unstable structure, studies based on its rapid transformation to its raw material O_2 show that O_3 application increases nitric oxide (NO) levels, decreases vasodilatation in ischemic areas, reduces hypoxia, activates superoxide dismutase (SOD), and decreases glutathione levels.[1,2]

In recent years, studies on IR injury have reported that medical O_3 treatment facilitates the recovery of diabetic foot wound, decreases claudication in peripheral arterial diseases by increasing blood flow, and reduces IR injury.[7-9] However, despite the increasing use of O_3 in medical areas, its effects on remote organ damage that develops in infrarenal abdominal aortic surgery are not clear. Therefore, in this study, we aimed to investigate the effect of O_3 on myocardial IR injury occurring after occlusion-reperfusion of infrarenal abdominal aorta in rats.

MATERIALS AND METHODS

This study was conducted at Süleyman Demirel University between July 2011 and February 2012. A total of 32 Wistar-albino rats (weighing 200-250 g) were obtained from Süleyman Demirel University Experimental Animal Production Laboratory. The rats were randomly and equally divided into four groups (n=8). They were kept in wire cages for 10 days in a circadian rhythm of 12 hours night and 12 hours day, at an ambient temperature of 24-26°C and a humidity of 50-60%. The rats were fed standard commercial pellets and city drinking water. Their food supply was cut off 12 hours before the study, but they were allowed water. The care of all rats was undertaken in accordance with the Principles for the Care of Experimental Animals formulated by the National Institute of Medical Research and the Guidelines for the Care and Use of Laboratory Animals prepared by the Laboratory Animal Resources Institute and published by the National Institute of Health (edition 85-23, revised in 1985). The study protocol and the experimental method were approved by the Süleyman Demirel University Ethics Committee (decision dated 17.02.2009 and numbered 01/08).

In the control group, laparotomy and IAA dissection were performed, but IAA occlusion was not undertaken. In the control+O_3 group, no additional intervention was undertaken apart from the specific procedures in the control group. In the aortic IR group, IAA was clamped and ischemia was performed for 60 minutes; then, the clamp was removed and reperfusion was undertaken for 60 minutes. The aortic IR+O_3 group underwent ischemia from IAA clamping for 60 minutes, followed by reperfusion for 60 minutes from declamped IAA. For 10 days, the control+O_3 and aortic IR+O_3 groups were intraperitoneally administered O_3 at a dose of 1 mg/kg/day prior to the procedure using an O_3 gas processor (Ozonosan Photonik 1014; Hansler GmbH, Iffezheim, Germany). The O_3 concentrations were monitored in real time with a ultra-violet spectrometer. Tygon polymer tubes and O_3-resistant disposable silicone-coated polypropylene syringes were used for the retention of O_3 and maintenance of the concentration during treatment.

All the rats were anesthetized with an intramuscular injection of ketamine hydrochloride (Ketalar Flakon®, Pfizer, İstanbul, Turkey) at a dose of 50 mg/kg and placed in the supine position under a heating lamp. The skin was aseptically prepared, and midline laparotomy was performed. To maintain the fluid balance, 10 mL of warm physiological saline solution was added to the peritoneal cavity. The intestines were pulled to the left with wet gas to access the abdominal aorta. A non-traumatic microvascular clamp (Vascu-Statt II, midi straight 1001-532; SCANLAN Int, St. Paul, Minnesota, USA) was placed in the IAA. The abdominal incision was kept closed to minimize the loss of heat and fluid. After 60 minutes of occlusion, the spinal cord was reopened, the microvascular clamp was removed from the IAA, and reperfusion was performed for 60 minutes. Aortic clamping was assessed by the loss of pulsation in the distal aorta during clamping and reperfusion was evaluated based on the recovery of pulsation after the removal of the clamp. Thus, the no-reflow phenomenon was prevented.

On completion of the reperfusion period, blood samples were taken from the right ventricle of the rats for biochemical analysis. All the rats were sacrificed under anesthesia, and their myocardial tissue samples were obtained. Some of the myocardial tissue samples were stored at -80°C until biochemical examination. The histopathological tissue samples were stored in a 10% formaldehyde solution until further analysis.

The rat tissue samples were washed with saline. The samples were homogenized in a cold phosphate buffer (pH=7.4) (Ultra-Turrax T25, Janke and Kunkel GmbH & Co., KG, Staufen, Germany) and the supernatants were sonicated for 30 seconds (Sonopuls UW 2070, Bandelin Electronic, Berlin, Germany). The protein contents of the supernatants were determined by the Lowry method.[10] The malondialdehyde (MDA), SOD, catalase (CAT) and myeloperoxidase (MPO) activities in the rat myocardial tissue samples were measured.

The MDA levels, the final product of lipid peroxidation, were determined by the double heating method of Draper and Hadley.[11] The absorbance of the resultant supernatant was measured at 532 nm spectrophotometer (Shimadzu UV-1601, Shimadzu, Kyoto, Japan). The MDA level was calculated by the absorbance coefficient of the MDA-thiobarbituric acid complex (absorption coefficient e: 1.56×10 cm^{-1}, M^{-1}) and the result was expressed as nanomoles of milligrams of protein.

The SOD activity was measured using the methods described by Spitz and Oberley[12] based on the xanthine oxidase reaction. The results were expressed as units per milligram (U/mg) protein.

The CAT activity was measured using the technique described by Aebi[13] based on the determination of the rate constant of the hydrogen peroxide (H_2O_2) fragmentation rate (s^{-1}, k). The results were expressed as U/mg protein.

The MPO activity is a sensitive marker of polymorphonuclear leukocyte accumulation in tissues. It was detected using H_2O_2-dependent tetramethylbenzidine oxidation catalyzed by MPO.[14] The MPO unit was expressed as ΔA/minute/g tissue.

All the samples were evaluated by the same pathologist blind to the experimental groups. Myocardial injury in the sections was evaluated by a semi-quantitative scoring system in which myocardial disorganization, myofibrillar edema, and myofibrillar

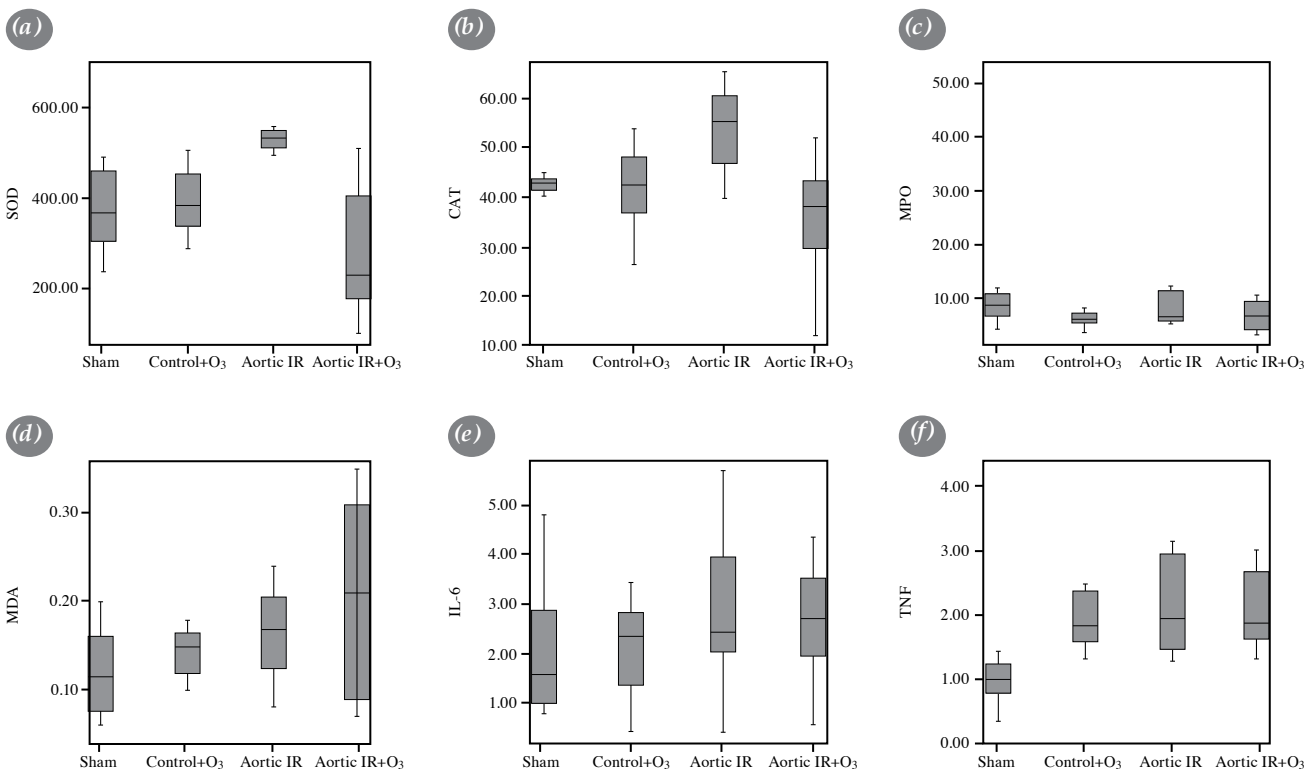

Figure 1. (a-f) Results of biochemical analyses for all groups.

O_3: Ozone; IR: Ischemia-reperfusion; SOD: Superoxide dismutase; CAT: Catalase; MPO: Myeloperoxidase; MDA: Malondialdehyde; IL-6: Interleukin-6; TNF: Tumor necrosis factor.

Table 1. Results of biochemical troponin-I in all groups

Group	Mean±SD	Min-Max	Significant differences between groups (p<0.05)
Sham	0.7813±0.04704	0.72-0.84	1-2, 1-3, 1-4
Control + O_3	0.8450±0.04690	0.79-0.91	2-3, 2-4
IR	2.3138±0.20486	2.02-2.59	3-4
IR + O_3	1.2775±0.16654	1.52-1.52	

Min: Minimum; Max: Maximum; SD: Standard deviation; O_3: Ozone; IR: Ischemia-reperfusion.

eosinophilia were scored as follows: (-) no change, (+) mild focal changes, (++) moderate multifocal changes, and (+++) significant widespread changes.[15]

Statistical analysis

The experimental data were analyzed using SPSS for Windows version 16.0 (SPSS Inc., Chicago, Illinois, USA). For normally distributed data, multiple groups were compared using a one-way analysis of variance followed by Tukey's post-hoc parametric tests, while the paired groups were compared using a Student's t-test. For non-normally distributed data and histopathological damage detected in heart, the Kruskal-Wallis test was used to compare multiple

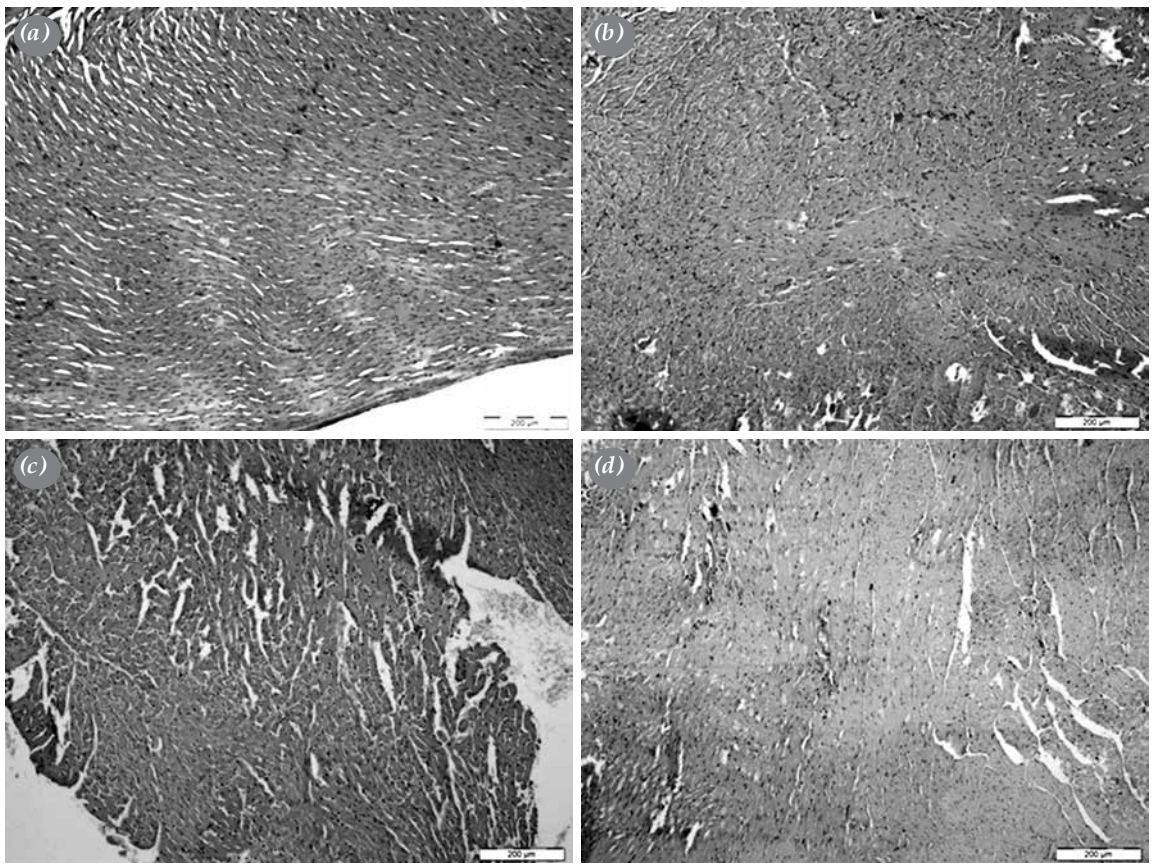

Figure 2. (a) Histopathological sample from control group; histologic grade 0 (H-E×200). **(b)** Histopathological myocardial sample from control + O_3 group: multifocal and moderate myocardial disorganization, swelling of myofibrils, and eosinophilia; histologic grade 1 (H-E×200). **(c)** Histopathological myocardial sample from aortic ischemia-reperfusion group: significant widespread myocardial disorganization, swelling of myofibrils, and eosinophilia; histologic grade 3 (H-E×200). **(d)** Histopathological myocardial sample from aortic+ozone ischemia-reperfusion group: mild focal myocardial disorganization, swelling of myofibrils, and eosinophilia; histologic grade 1 (H-E×200).

groups and the Mann-Whitney U test was used for paired comparisons based on non-parametric methods. A *p* value <0.05 was considered statistically significant in analyses.

RESULTS

Figure 1a-f and Table 1 present the results of the levels of MDA, SOD, CAT, MPO, interleukin-6 (IL-6), tumor necrosis factor (TNF), and troponin-I in the myocardial and serum samples of all groups. Compared to the control group, in the aortic IR group, the values of SOD (538.7±36.2 vs. 374.1±92.8, p=0.001) and CAT (54.0±9.2 vs. 42.8±1.5, p=0.036) were significantly higher (p<0.05). The O_3 administration in the aortic IR+O_3 group resulted in a significant decrease in the SOD (278.7±148.4 vs. 538.7±36.2, p=0.002) and CAT (35.9±12.5 vs. 54.0±9.2, p=0.006) values compared to the aortic IR group (p<0.05). Troponin-I was found to be significantly higher in the aortic IR group compared to the control group (2.3±0.2 vs. 0.8±0.0; p<0.05). There was a significant decrease in the troponin-I levels in the aortic IR+O_3 group following O_3 treatment compared to the aortic IR group (1.3±0.2 vs. 2.3±0.2). There was no statistically significant difference between the groups in terms of MPO, MDA, IL-6 and TNF results (p<0.05).

Histopathologically, the sham group was evaluated as grade 0, figure 2a. Significant widespread myocardial disorganization, swelling in myofibrils and eosinophilia findings (histologic grade 3) were found to be significantly higher in the aortic IR group than in the control group (2±0.5 vs. 1.4±0.5, p=0.036) (Figure 2b, c). In the aortic IR+O_3 group, there was a decrease in mild focal myocardial disorganization, swelling of myofibrils and eosinophilia damage (histologic grade 1) compared to the aortic IR group; however, this difference was not statistically significant (1.6±0.5 vs. 2±0.5, p<0.05) (p=0.175) (Figure 2c, d). Furthermore, there was no statistically significant difference between the control group and the aortic IR+O_3 group in terms of histopathological findings of myocardial disorganization, swelling of myofibrils, and eosinophilia (1.4±0.5 vs. 1.6±0.5, p=0.333) (Figure b, d).

DISCUSSION

The results of this study showed that O_3 had protective effects on myocardial damage caused by aortic IR in rats. The following three findings after O_3 administration support this hypothesis: *(i)* significantly decreased SOD and CAT levels, *(ii)* significantly decreased plasma troponin-I levels, and *(iii)* decreased histopathological changes associated with myocardial injury, albeit not significant.

Narin et al.[16] reported increased SOD and CAT levels in the myocardial tissue samples of the aortic IR group compared to the control group. Similarly, in the current study, the SOD and CAT levels in the myocardial tissue of the aortic IR group were found to be significantly higher compared to the control group.

Moreover, León et al.[17] stated that controlled O_3 administration could reduce the damage induced by reactive oxygen species (ROS) production through oxidative preconditioning or adaptation to oxidative stress. Similarly, Chen et al.[18] reported significantly reduced SOD and CAT levels as a result of preconditioning with O_3 in experimental renal IR injury in rats. Parallel to these reports, we also detected a significant decrease in the SOD and CAT levels in the myocardial tissue of the aortic IR+O_3 group compared to both control and aortic IR groups.

Since O_3 is denser and has higher resolution than O_2, it increases plasma erythrocyte tissue oxygenation through ROS, immunostimulation, release of platelet-mediated growth factor, and endothelium-mediated NO through lipid oxidation, bone marrow stem cell activation, and resistance to the oxidant process.[6,19,20]

Ozone first reacts with thiol compounds, enzymatic SOD, CAT, and glutathione peroxidase compounds containing unsaturated fatty acids and non-enzymatic sulfhydryls in the plasma. ROS derivatives, such as superoxide (O_2-) and H_2O_2, are formed against the oxidizing activity of O_3.[21] At this point, the main effects of O_3 are to increase the erythrocyte 2,3-diphosphoglycerate as a secondary messenger through H_2O_2 that results from unsaturated fatty acid oxidation of the erythrocyte membrane, to shift the hemoglobin-O_2 dissociation curve to the right, and consequently to increase tissue perfusion.[4,22] In parallel with tissue perfusion, the production of hemo-oxidase^{-1} and heat shock protein-70 are increased, leading to the inhibition of tissue damage and an antiinflammatory effect.[3,4,23,24] Hence, it should be noted that for O_3 to create a positive effect, there is a two-directional activity; on one side ROS radicals, such as SOD and H_2O_2 are formed, and on the other side, there should also be sufficient increase in the antioxidant capacity in order to remove these products, and the negative effects on IR; e.g., the increased compensatory tissue perfusion pathways should be eliminated. Therefore, a possible hypothesis is that O_3 attempts to maintain its defense mechanism; i.e., the ROS response, within physiological limits.

Based on all these mechanisms of action, it has been suggested that there may be a cut-off line between the level of oxidative stress and the efficacy or toxicity of O_3 therapy.[4] Sagai and Bocci[4] stated that the amount of plasma antioxidant capacity in basic O_3 activity is important. In addition, the authors advocated that total antioxidant capacity decreased in the first 20 minutes in direct proportion to the dose; however, lipid peroxidation products, such as MDA, and oxidized glutathione increased. In the current study, in accordance with the literature, we also found that MDA, the final product of lipid peroxidation, was elevated in the aortic IR+O_3 group compared to the aortic IR group; however, this was not statistically significant. At the same time, similar to the literature, compared to the aortic IR group, the O_3 treatment resulted in increased MDA levels of the aortic IR+O_3 group, in response to increasing lipid peroxidation, but this increase was, again, not statistically significant. At this point, the application of O_3 in the presence of IR increased the lipid peroxidation product MDA in the myocardial tissue, and possibly as a response, antioxidant enzymes; i.e., SOD and CAT were consumed. It seems that O_3 created enough oxidant products in the tissue, and antioxidant enzyme production and consumption were stimulated.

Merin et al.[25] showed that myocardial injury was reduced through the administration of O_3 before and after reperfusion in myocardial IR. Di Filippo et al.[26] reported that O_3 reduced immunological, apoptotic, inflammatory and oxidative damage caused by myocardial IR. The authors attributed this to the ischemic preconditioning mechanism of O_3 through NO, which decreased the damage caused by ROS via the antioxidant system. In the current study, the plasma troponin-I values were found to statistically significantly decrease in the aortic IR+O_3 group compared to the aortic IR group. Although we did not evaluate NO in the current study, we consider that as a result of increased vasodilation and myocardial perfusion due to the possible increase in NO, the troponin-I values of the aortic IR+O_3 group was significantly lower than those of the aortic IR group. We also think that increased myocardial perfusion due to the release of NO improved histopathological findings in the aortic IR+O_3 group compared to the aortic IR group.

Furthermore, Di Filippo et al.[26] administered different doses of O_3 to myocardial IR rats and reported that although 100 μg/kg O_3 did not result in a significant reduction in myocardial inflammatory cytokine IL-6, immunocyte CD4 and CD8 levels, these levels were found to significantly decrease at doses of 150 μg/kg and 300 μg/kg. We did not detect a significant decrease in the IL-6 and TNF levels in the current study, which can be explained by the low dose of O_3 administered (100 μg/kg).

In this study, in the aortic IR group, significant widespread myocardial disorganization, swelling in myofibrils, and eosinophilia were detected, and in the IR+O_3 group, morphological changes associated with myocardial damage were histopathologically reduced (Figure 2c, d); however, no statistically significant difference was found between the two groups. Di Filippo et al.[26] suggested that the myocardial caspase-3 enzyme, known as the endothelial enzyme in the apoptosis mechanism, decreased with the O_3 administration depending on the dose, and O_3 inhibited apoptosis and decreased the infarct area.

In conclusion, this study showed that in myocardial injury in the experimental infrarenal aortic ischemia-reperfusion model, the tissue superoxide dismutase and catalase, and serum troponin-I levels statistically significantly decreased in the aortic ischemia-reperfusion+ozone group compared to the ischemia-reperfusion group. Furthermore, the histopathological examination revealed reduced myocardial findings despite not being statistically significant. Further experimental studies are needed to identify the effect mechanism and the hemodynamic effects of ozone in detail through nitric oxide and an organ bath. We consider that following these experimental studies, the effect of ozone on myocardial injury in infrarenal abdominal aorta surgery can also be clinically investigated.

Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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