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Original Article

Protective effects of ozone therapy against lower extremity ischemia–reperfusion injury in extremity muscles of rats ischemia-reperfusion and ozone

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Abstract

Aim: Ischemia-reperfusion injury (IRI) results in oxidative stress. The present research investigates ozone administration in rats IRI through histopathological and biochemical assessments.

Material and Methods: We assigned 24 rats, Wistar albino, into four randomized groups (n = 6 each): S, O, IRI and IRI + O. The aorta was clamped for 45 minutes, and reperfusion was followed by 120 minutes to induce IRI. Ozone (0.7 mg/kg, intraperitoneal) was administered before the reperfusion period in the IRI + O and without ischemia in the O. Muscle tissues were evaluated histopathologically in terms of neutrophil infiltration, hemorrhage, interstitial edema, and myocyte damage. In addition, biochemical analyses were performed to measure malondialdehyde (MDA) and glutathione (GSH) levels.

Results: Remarkably elevated MDA values were found in the IRI than the S and O. ($p=0.007$ and 0.006, respectively). Although ozone reduced MDA in the IRI + O, this reduction did not meet statistical significance. GSH values were considerably decreased in the IRI according to the S and O ($p=0.010$ and $p=0.001$, respectively); but, they were elevated in the IRI + O than the IRI ($p=0.007$). Histopathology demonstrated significantly reduced hemorrhage, neutrophil infiltration, muscle injury, and interstitial edema in the IRI + O than the IRI ($p<0.001$, $p=0.037$, $p=0.042$, and $p=0.029$, respectively).

Conclusion: Ozone therapy mitigates IR–induced skeletal muscle injury by reducing histological alterations and enhancing antioxidant defenses in rats. These findings suggest ozone as a potential agent in IRI management; however, further research is required to optimize dosing and timing.

Keywords: Ischemia–reperfusion injury, ozone therapy, skeletal muscle, oxidative stress, rat

INTRODUCTION

Peripheral artery disease (PAD) affects millions of individuals worldwide. As a result of the advances in peripheral arterial surgery, many patients at risk of amputation caused by PAD gain the chance of surgical revascularization [1,2]. However, these advancements in surgical area lead clinical issues related to ischemia-reperfusion injury [2]. Acute limb ischemia is a condition in which blood flow of the extremity is interrupted causing varying degrees of injury in the extremities majorly depending on the duration and the extent of the occlusion [2]. Acute limb ischemia may be encountered during routine vascular or orthopedic surgical interventions and may also appear as

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result of many other conditions such as trauma or shock [3]. Due to these conditions and also other various etiologies, including acute thromboembolic events, atherosclerotic plaque complications, hypercoagulability, hyperviscosity, tissue blood flow is disrupted [4,5]. After the restoration of this reduced blood flow with techniques such as embolectomy, thrombolytic application, catheter-mediated interventions, and peripheral bypass surgery, local and systemic tissue damage results from reactive oxygen species production [2]. Local complications occuring as a consequence of reactive oxygen species production are tissue edema, which may lead to compartment syndrome, and tissue necrosis, which develops as a result of immune-mediated tissue damage and may lead to loss of extremities. On the other hand, systemic complications can be listed as the development of rhabdomyolysis; multiorgan failure in the form of acidosis, hyperkalemia, hypercalcemia, renal failure, liver failure, pulmonary edema, arrhythmia, and cardiac arrest [2,3].

Local and systemic inflammatory responses are known to contribute to tissue damage [6]. It is accepted that these responses are triggered by reactive oxygen species, which occur in damaged tissues due to the increased activities of xanthine oxidase, NADPH oxidase, the electron transport system, and the nitric oxide synthase enzyme [7]. Thus, treatment modalities are tried to be generated by targeting these systems. Establishing experimental animal models is crucial for understanding the mechanism of ischemia-reperfusion injury and evaluating potential therapeutic options [7,8].

Ozone is a highly responsive oxidative chemical formed by three oxygen atoms [9]. Identified as a disinfectant, ozone was used in the management of gangrene during World War 1 [10]. Ozone treatment has been widely utilized in various pathogenic conditions, including dermatological, urological, diabetes-related complications, dental problems, cardiovascular and cerebrovascular disorders, and malignancies, as a result of years of research aimed at defining its precise mechanism [9]. Similarly, numerous manuscripts have highlighted the relationship between ozone and IRI causes from various diseases in the literature [10-13].

In our current research, we wanted to investigate the effect of ozone on biochemical and histopathological parameters associated with IRI.

MATERIAL AND METHODS

Ethical Approval and Animals

This experimental study was conducted at the Gazi University Animal Experiments Laboratory in accordance with the ARRIVE guidelines. The study protocol was approved by the Animal Research Committee of the Gazi University (G.Ü.ET-24.013,with 2024/03/01), Ankara, Turkey. All the animals were maintained in accordance with the recommendations of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Our research involved twenty-four male Wistar Albino rats randomly divided into four groups, each weighing between 250 and 350 grams.

All rats were kept in a controlled environment with a 12/12-hour light/dark cycle, maintaining a temperature of 22-24 ºC and humidity levels of 45-55%. They had unrestricted access to a classic pellet diet and water until the experiment day.

Induction of Hind Limb Ischemia Reperfusion

Following intramuscular injection of 5 mg/kg xylazine and 45 mg/kg ketamine, to regulate their body temperature during the surgical procedures,the subjects were positioned on a heating pad. Body temperature was monitored continuously using a rectal thermometer and maintained between 36.5 and 37.5 °C with the aid of a thermostatically controlled heating pad during both ischemia and reperfusion periods.Subjects were randomly assigned into 4 equal groups (n=6) as group sham (S), group ozone (O), group ischemia-reperfusion (IRI), and group ischemia-reperfusion with ozone treatment (IRI+O).

In S, just laparotomy was performed and the abdominal aorta was dissected. Animals were sacrificed 120 minutes later following the 0.3 ml intraperitoneal injection of 0.9% NaCl at the 45th minute of experiment. Same procedures were followed for the animals in O, with the exception of 0.7 mg/kg intraperitoneal ozone application instead of salin. In IRI, following laparotomy, the infrarenal aorta was occluded with a nontraumatic vascular clamp for 45 minutes. At the end of the ischemia period (at the 45th minute of the process) 0.3 ml intraperitoneal injection of 0.9% NaCl was applied after which reperfusion was initiated for the next 120 minutes by releasing the clamp. An identical protocol as in IRI was performed for the animals in IRI+O; but unlike the IRI, 0.7 mg/kg ozone rather than salin was administrated intraperitoneally at the 45th minute of the process. After ozone administration, vascular clamp was removed for reperfusion period lasting 120 minutes.

At the completion of the reperfusion, rats from each team were euthanized, and right gastrocnemius muscle tissues were harvested and organized for biochemical and histopathological analyses.

Biochemical Analysis of Muscle Samples

For MDA and GSH levels measurement in the muscle samples, half of the right gastrocnemius muscles were harvested and stored at -80°C.

MDA levels were analyzed using the TBARS method [14]. For the measurement of tissue MDA levels, 450 mg of cold trichloroacetic acid (TCA) solution was combined with 50 mg of muscle tissue and homogenized on ice. At 4500 rpm for 15 minutes, these homogenates were centrifuged. The supernatants were transferred to clean Eppendorf tubes, and following a second centrifugation for 10 minutes, the samples were put into

glass tubes. Butylhydroxy toluene (BHT) and thiobarbituric acid (TBA) were added to the samples in glass tubes. After vortexing the mixture for 20 seconds, the tubes were capped and then left in a water-bath adjusted at 100 °C for 15 minutes. After a final centrifugation at 4500 rpm, the absorbance of the samples in the 96-well plate was measured at 535 nm. Distilled water was used as a blank and treated as a sample.

GSH, an endogenous antioxidant, was measured according to the modified Ellman procedure [15]. To that end, 50 mg of muscle tissue samples were homogenized within the 450 mg cold TCA solution. After that, the homogenates were centrifuged, and dithiobisnitrobenzoate (DTNB) and 0.3 M disodium hydrogen orthophosphate dihydrate (Na2HPO4-2H2O) solution were added to the supernatant. All samples were put into a 96-well plate and incubated for 10 minutes at room temperature. The samples' absorbance was measured at 412 nm using a spectrophotometer. Distilled water was used for the blank and was treated as the sample.

Histopathological Analysis of Muscle Samples

Muscle samples were immersed in neutral buffered 10% formalin immediately after removal from the animals. Following fixation, muscle samples were processed for paraffin embedding. For histopathological investigation, four slices of 4 µm thickness, separated by a 50 µm spacing, were excised from paraffin blocks utilizing a HistoCore MULTICUT microtome (Leica, Germany) and then stained with hematoxylin and eosin (H&E). These samples were examined and assessed at 200× and 400× magnifications using a computer-assisted light microscope (Leica DM 4000B, Germany), with pictures taken using Leica LAS V4.12 software.

Muscle injury resulted from ischemia- reperfusion was evaluated based on the histopathological changes involving hemorrhage, neutrophil infiltration, myocyte damage (disorganization, swelling and degeneration of the muscle fibers) and interstitial edema. All parameters were graded in a scale in which 0 indicated normal appearance of muscle tissue, 1 indicated mild injury, 2 indicated moderate injury and 3 indicated severe injury. Beside individual assessment and comparison of the histopathological changes, sum of the scores of all criterions ranging between 0 and 12 was also determined considering the total muscle injury score and compared between the groups [16,17].

Statistical Analysis

SPSS 26.0 for Windows, developed by SPSS (Chicago, IL, USA) was used to compile our statistical data. The results were reported as the mean ± standard error (SE). Biochemical and histological parameters were assessed using the Kruskal-Wallis test, the Mann-Whitney U with Bonferroni correction, and the ANOVA test. Statistical significance was accepted as a p-value less than 0.05.

RESULTS

Results of Biochemical Analysis

MDA levels were observed to be substantially different across the groups (p=0.028). In the S and the O , MDA levels were found to be similar (p>0.05). In IRI, there was a significantly higher level of MDA compared to the S and the O (p=0.007 and p=0.006, respectively). Although MDA levels decreased in the IRI+O, no substantial disparity was observed when compared to the IRI (p > 0.05) (Figure 1).

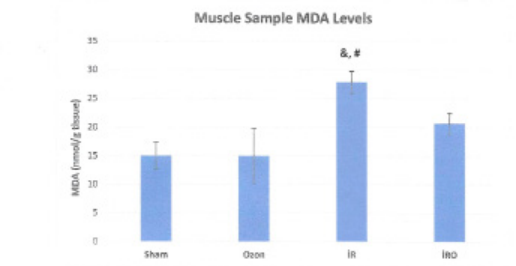

Figure 1. Muscle tissue MDA levels

&: Significant difference (p<0.05) compared to the Sham group by Kruskal Wallis and Bonferroni-corrected Mann-Whitney U test.
#: Significant difference (p<0.05) compared to the Ozone group by Kruskal Wallis and Bonferroni-corrected Mann-Whitney U test.

A noticeable variance in muscle tissue GSH levels was noticed in the groups (p=0.023). GSH levels were similar in the S and the O (p>0.05). GSH levels in the IRI were remarkably reduced compared to the S and the O (p=0.010 and p=0.001, respectively). Additionally, tissue GSH levels were significantly increased in the IRI+O compared to the IR (p=0.007) (Figure 2).

Figure 2. Muscle tissue GSH levels

&: Significant difference (p<0.05) compared to the Sham group by Kruskal-Wallis and Bonferroni-corrected Mann-Whitney U test.
#: Significant difference (p<0.05) compared to the Ozone group by Kruskal-Wallis and Bonferroni-corrected Mann-Whitney U test.
%: Significant difference (p<0.05) compared to the IRI group by Kruskal Wallis and Bonferroni-corrected Mann-Whitney U test.

Results of Histopathological Analysis

Through the assessments of histopathological findings, the scores for hemorrhage, neutrophil infiltration, disorganization and degeneration of the muscle fibers, and interstitial edema exhibited significant differences among the groups (p<0.001, p=0.016, p<0.001, and p=0.004, respectively) (Table 1).

Table 1. Muscle tissue histopathological data (Mean±SE)

Parameter	Group C (n=6)	Group I/R (n=6)	Group S-I/R (n=6)	P**
Muscle atrophy-hypertrophy	0.33±0.21*	1.50±0.22	0.67±0.21*	0.015
Muscle degeneration- congestion	0.33±0.21	1.33±0.42	0.67±0.21	0.085
Internalisation of muscle nuclei -oval/central nucleus	0.17±0.17*	1.50±0.22	0.83±0.31*	0.012
Fragmentation -hyalinization	0.33±0.21	1.50±0.42	0.83±0.31	0.070
Leukocyte cell infiltration	0.50±0.22	1.17±0.31	0.33±0.21	0.075

C: Control, I/R: Ischemia/Reperfusion; S-I/R:Silymarin- Ischemia Reperfusion; P **Significance level with Kruskal Wallis test p <0.05; *p<0.05: Compared to Group I/R

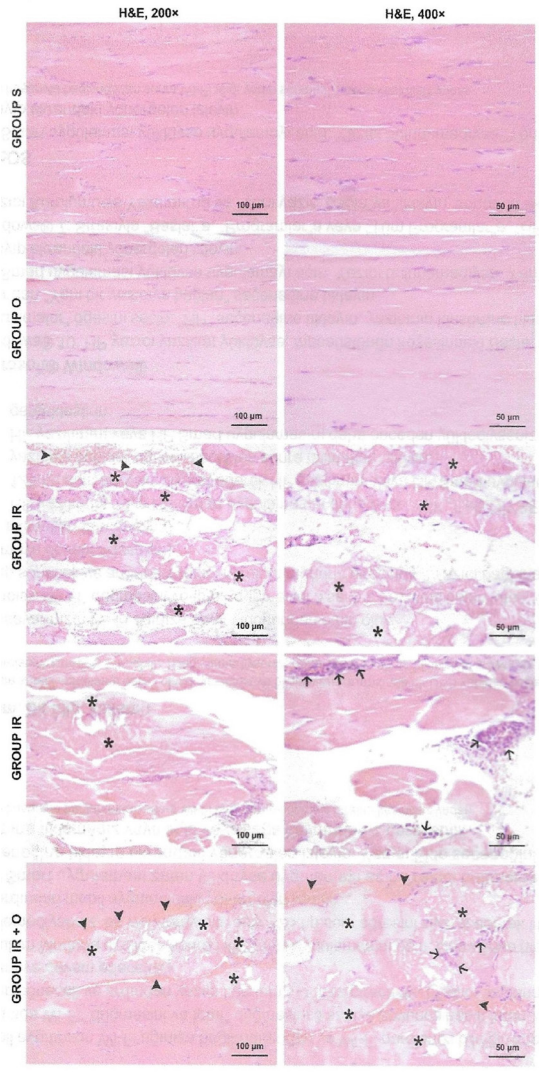

Figure 3. The muscle sections from the specimens of each group.

Hemorrhage (arrowheads) was obvious in some regions of the muscle specimens of the IRI group, while it was limited to fewer regions around the injured muscle in the specimens of the IRI+O group. Neutrophils (arrows) infiltrating the sites of injured muscle, were found to be less in the specimens of the IRI+O group than in the sections from the IRI group. Also, muscle fibers with varying degrees of injury, some of which exhibiting swelling and cytoplasmic fragmentation (asterisks), were more extensive in the IRI compared to the IRI+O .

Hemorrhage in the IRI was more obvious compared to the S and the O (p<0.001, both); while it was less prominent in the IRI+O than the IRI (p<0.001), (Table 1, Figure 3).

Neutrophil infiltration was notably greater in the IRI than in the S and the O (p=0.037 and p=0.002, respectively). However, neutrophil infiltration in the IRI+O was significantly milder compared to the IRI (p=0.037), (Table 1, Figure 3).

Disorganization and degeneration of the muscle fibers in the IRI was considerably more severe when compared to the S and the O (p<0.001 and p=0.017, respectively). Likewise, disorganization and degeneration of the muscle fibers in the IRI+O was more severe compared to the S and the O (p<0.001 and p=0.017, respectively); nevertheless, it was remarkably milder compared to that in the IRI (p=0.042), (Table 1, Figure 3).

Interstitial edema was significantly more excessive in the IRI compared to the S and the O (p=0.002 and p<0.001, respectively). Nonetheless, interstitial edema in the IRI+O was considerably milder than in the IRI (p=0.029), (Table 1, Figure 3).

The total muscle injury showed differences across the groups (p < 0.001). It was higher in IRI than in the S and the O (p < 0.001, both). Similarly, the total injury score of the IRI+O was higher than that of the O (p=0.029); however, it was remarkably lower compared to the IRI (p<0.001) (Table 1).

DISCUSSION

We wanted to examine the protective effects of ozone on IRI in rat muscle tissue with this experimental study.

As hypothesized, ozone administration led to a reduction in histopathological alterations such as hemorrhage, interstitial edema, neutrophil infiltration, and muscle fiber degeneration, while enhancing antioxidant defense mechanisms, was reflected by increased GSH levels and reduced MDA levels.

Ischemia-reperfusion injury is a multifactorial process involving oxidative stress, inflammation, and tissue degeneration. The abrupt reintroduction of blood flow leads to excessive production of reactive oxygen species (ROS), which promotes protein oxidation, lipid peroxidation, and cellular injury [4-6,18]. In our study, significantly elevated MDA levels in the IRI and markedly reduced GSH levels confirmed the presence of oxidative stress,

which is consistent with prior literature [4,19]. Importantly, ozone administration restored GSH levels and partially reduced MDA levels, although the latter did not reach statistical significance. These results suggest that ozone therapy modulates redox homeostasis and may enhance endogenous antioxidant capacity, as proposed by previous biochemical studies [10,12,20].

Our histopathological findings corroborate the biochemical outcomes. Hemorrhage, neutrophil infiltration, and interstitial edema—key indicators of inflammatory tissue damage—were significantly attenuated by ozone in the IRI+O compared to the IRI. These results align with prior studies demonstrating ozone's anti-inflammatory and tissue protective properties in models of myocardial [10,12] and skeletal muscle IRI [13,21]. One of prior studies by Seven et al. demonstrated that ozone therapy had therapeutic efficacy in a rat muscle strain model, even superior to non-steroidal anti-inflammatory drugs, by reducing edema and inflammatory infiltration [21]. Similarly, our findings support ozone's potential role in reducing local muscle damage in the setting of reperfusion.

In the present study, infrarenal aortic clamping was preferred to induce hind limb ischemia-reperfusion because this method is widely accepted and provides a standardized, reproducible model in rats. Selective clamping of the iliac or femoral arteries may yield variable ischemic responses due to collateral circulation, whereas infrarenal aortic occlusion ensures consistent ischemia in both extremities [16,17]. Although transient ischemia of abdominal organs such as intestines might theoretically occur, our analyses were exclusively focused on gastrocnemius muscle tissue. Both the biochemical markers (MDA and GSH), which are validated indicators of oxidative stress and antioxidant defense in skeletal muscle, [14,15,19], and the histopathological parameters (hemorrhage, neutrophil infiltration, interstitial edema, myofiber degeneration) specifically reflect local muscle injury. Therefore, the results presented in this study reliably demonstrate the effects of ozone on skeletal muscle I/R injury, independent of possible intestinal involvement.

Several mechanisms may underlie the observed protective effects of ozone. At controlled doses, ozone is believed to trigger a mild oxidative stimulus that activates the Nrf2/ARE pathway, promoting the upregulation of antioxidant enzymes. [20,22] This preconditioning effect results in increased tissue resilience against oxidative damage during reperfusion. The significant increase in GSH levels in our the IRI+O supports this mechanism. Moreover, previous work has suggested that ozone modulates nitric oxide metabolism, reduces pro-inflammatory cytokine release, and stabilizes mitochondrial function—all of which contribute to improved tissue viability during IRI [9,11,20].

While these findings are promising, our study has limitations. The limited sample size (n=6 per group) may restrict the generalizability of the findings. The number of subjects in each group (n=6) was chosen in accordance with previous ischemia-reperfusion studies employing similar models, which demonstrated that this sample size is adequate to detect significant differences in oxidative stress and histopathological outcomes [16,17,19]. Although no formal a priori power analysis was performed, the chosen group size balances statistical validity with the ethical principle of reduction in animal research.

Second, histopathological evaluation was semi-quantitative and subject to observer variability. Third, systemic effects of ozone on other organs (e.g., the kidney, liver) were not evaluated. Lastly, although our ozone dose (0.7 mg/kg) was based on previous studies [13, 20], the optimal dose and timing for maximal therapeutic benefit remain to be determined. The ozone regimen in our model (0.7 mg/kg, intraperitoneal, administered immediately before reperfusion) was selected a priori from preclinical I/R literature indicating that low-dose ozone exerts a hormetic preconditioning effect—enhancing antioxidant defenses and mitigating histological injury—when applied before or at the onset of reperfusion in skeletal muscle and myocardium [10,12,13,20]. Although medical ozone is used in humans via oxygen–ozone mixtures (e.g., autohemotherapy or local applications), a guideline-level dosage specifically for acute limb I/R has not been established; thus, our dosing aligns with animal data and highlights the need for translational dose-finding studies [9,22].

In this study, we deliberately adopted a single, pre-reperfusion ozone dose to interrogate the peri-reperfusion therapeutic window with minimal exposure. While preclinical reports also describe brief pre- or postconditioning courses to prolong cytoprotection, the optimal duration and frequency remain undefined and should be determined by translational dose-finding studies. Current evidence supports low-dose, intermittent administration consistent with a hormetic mechanism [10,12,13,20,22].

Clinically, ozone therapy is regarded as safe when used at controlled medical doses and delivered via approved methods such as autohemotherapy or local infiltration. Reported side effects are usually mild and transient, including local discomfort or fatigue, while severe complications are rare and generally associated with improper technique. Importantly, inhalation of ozone is contraindicated due to pulmonary toxicity risk. Thus, although our findings support a protective role in skeletal muscle I/R, further clinical studies are required to validate both efficacy and safety in this specific context [9,22].

CONCLUSION

In conclusion, our study demonstrates that ozone therapy alleviates skeletal muscle damage associated with ischemia-reperfusion injury by enhancing antioxidant capacity and reducing histopathological alterations. The findings enhance the existing evidence regarding the therapeutic potential of ozone in vascular and surgical conditions associated with reperfusion injury.

Additional research is necessary to clarify the molecular pathways involved and to investigate therapeutic applications in vascular surgery and trauma.

Ethics Committee Approval: It was received from Gazi University Animal Experiments Local Ethics Committee, under code number G.Ü.ET-24.013.

Patient Consent for Publication: Not necessary for this manuscript.

Data Sharing Statement: The information in the present research are contained within this published paper.

Author Contributions: ZY, AK, AÖ, and MA developed the study design and conducted data analysis and interpretation. AÖ, BK, and HD conducted the experiments. MA and AK verify the authenticity of all raw data. AK, ET, ZY, and BK contributed scientific and technical support and conducted a critical review of the article for significant intellectual content. HD and BK gathered samples. AK, ET, and ZY conducted histological and biochemical experiments. All authors have reviewed and consented to the final paper.

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